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STUDIES ON HERPES SIMPLEX VIRUS

I. AN ANTIGENIC ANALYSIS OF FOUR STRAINS OF VIRUS ISOLATED FROM A HUMAN SUBJECT

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Despite accumulated data in the literature dealing with the immunological pattern of recurrent herpes in man, the pathogenesis of this common affliction remains obscure. The causes of the contrasting refractoriness to herpetic infection of non herpetics and the continuing susceptibility to focal infection of individuals apparently harboring herpes virus and its circulating antibody concomitantly are still subject to speculation. The precise role of specific humoral antibodies in recurrent herpetic attacks, whether of preventing the spread of virus from superficial tissues, or of determining by periodic fluctuation in titer the appearance of eruptions, or of exerting a more intricate influence upon the host cell-virus relationship, is undecided. The present series of experimental studies, limited to the laboratory, were made in an effort to elucidate some of the factors concerned in the infective activity of herpes virus.

This communication reports experimental findings with regard to: (a) the quantitative relationship between herpes simplex virus and its neutralizing antibody, (b) the comparative capacity of interval serum samples from a herpetic individual to neutralize successive autologous strains of virus from concomitant recurrent vesicles, and (c) the determination of the antigenic relationship of successive isolates by employment of strain-specific antiherpetic rabbit serums for cross-neutralization tests in mice.

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* The material in this paper represents in part the thesis submitted by G. A. Garabedian in partial fulfillment of the requirements for the Ph.D. degree.

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MATERIALS AND METHODS

Virus strains.—Capital letters designate virus strains; small letters identify antiserum samples, with the letter indicating the virus strain giving rise to antibody.

O.—Isolated by one of us (J.T.S.) in 1946 from a human patient with aphthous stomatitis, conjunctivitis and balanoposthitis, and maintained since through 13 intracerebral passages in mice.

M14.—Isolated in 1952 from a recurrent herpetic lesion on the lower lip of an adult man. When first employed in this study the strain was in its 14th mouse brain passage with a log LD_{50} of -4.0, as tested in 8- to 9-week-old Swiss albino mice, CFW strain, by intracerebral injection of 0.03 ml.

A, B, C, D.—Isolated in 1952 from single or multiple vesicles of a male patient (G.G.) at respective intervals of 48, 24, and 26 days. A positive history for the donor of an initial attack of aphthous stomatitis was doubtful. Vesicular circumoral lesions appeared repeatedly in the patient throughout the year, especially in winter and after infection with a common cold.

Serums.—Serum samples *a*, *b*, *c*, and *d* were obtained from patient G. G. at the same time that virus strains A to D were isolated, respectively. Sample *e* was taken 3 months after appearance of the last vesicles in the series.

Anti-A, anti-B, anti-M14 and anti-O represent the pooled serums obtained from groups of 3 rab-

TABLE 1.—*The LD_{50} of strain O herpes simplex virus measured in mice of different age and weight.*

Age in days	Mean weight in g	Negative log LD_{50}/0.03 ml*
21	29.4	4.67
28	41.6	4.67
35	62.0	5.00
45	89.4	4.50

* Four mice were employed to test each successive dilution of virus.

bits bled 6 weeks after corneal infection with virus strains A, B, M14 or O, respectively.

All serums were stored frozen at −70 C.

Cross-neutralization tests.—Successive decimal dilutions of infected mouse brain and serial 2-fold dilutions of serum were prepared. The serum was not inactivated. Volumes of 0.2 ml each of serum and virus dilutions were mixed, incubated for 1 hour at 37 C, and stored for 5 hours at 4 C. Each mixture was tested in 5 mice by intracerebral injection of 0.03 ml. The animals were observed for 12 days, death being attributed to the virus when it occurred within 48 hours from inoculation or later. The neutralizing capacity of the serum was assessed by the PD_{50}, the reciprocal of the dilution calculated by the method of Reed and Muench[1] to protect 50% of mice from death.

Infectivity tests.—Serial 10-fold dilutions of virus in nutrient broth were tested by intracerebral inoculation into mice of 0.03 ml samples. The LD_{50} was calculated by the method of Reed and Muench.

RESULTS

1. The ratio of herpes simplex virus and its antibody in neutral admixtures

As a first approach the stability of the LD_{50} as a measure of herpes virus infectivity was investigated. In procedures for the determination of LD_{50} titers or neutralization indexes it has been customary to keep constant the indicator host, route of inoculation, source of virus, and the virus dosage. Little attention has been directed to the average weight and age of the inoculated animals. The influence of these factors on the LD_{50} was surveyed in mice 21, 25, 35 and 45 days of age, respectively, by the injection of strain O virus into successive groups of 4 mice for each dilution of virus. The mortality was recorded daily for 12 days. The results are given in table 1. It is apparent from these results that the variation in LD_{50} titer manifested by strain O virus on intracerebral inoculation falls within the usual limits of error, despite diversity in host weight and age.

The quantitative relationship between herpes simplex virus, measured by LD_{50}, and its neutralizing antibody, measured by PD_{50}, was examined in neutralization tests conducted in mice. Strains O and M14 and specific rabbit antiserums (anti-O and anti-M14) were employed. The results shown in table 2 indicate that within the limits of virus activity tested (10^{-1} to 10^{-4}), a linear relationship exists between the quantity of virus and the serum PD_{50} titer. Thus, each 6-fold change in the PD_{50} titer of the test antiserum reflects a 10-fold change in the dilution of virus. This relation was used in following studies for the correction of PD_{50} titers of serums tested with virus strains differing in potency.

2. The relationship between virus strains isolated successively from a single human subject and corresponding samples of homologous antibody

For investigation of the relationship to recurrent herpes of fluctuations in antibody and/or alterations in the antigenic constituents of the virus, a single human subject provided strains of herpes virus (A to D) from his vesicular herpetic lesions, and samples of serum (*a* to *d*) taken concomitantly. The virus and serum samples were secured immediately upon the onset of each of four successive recurrent attacks of herpes simplex. A fifth serum sample (*e*) was

1. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**: 493–497.

TABLE 2.—*The determination of the ratio between amounts of herpes simplex virus and its antiserum in neutral admixtures tested in mice.*

Virus strain	Anti-serum	Negative log LD_{50}	Serum PD_{50}*	Serum factor: 6^n	$PD_{50}/6^n$
O	anti-O	1	32	6^1	5.37
		2	186	6^2	5.17
		3	1259	6^3	5.83
		4	6123	6^4	4.72
M14	anti-M14	1	4.5	6^1	0.75
		2	28	6^2	0.78
		3	187	6^3	0.86
		4	1024	6^4	0.79

* The reciprocal of the serum dilution calculated by the method of Reed and Muench[1] to protect 50% of mice from death after inoculation of 0.03 ml of the serum-virus mixture intracerebrally.

obtained 3 months later. The serums were tested with the virus isolates by cross-neutralization in mice. The serum PD_{50} titers were corrected as previously noted. Table 3 summarizes the results of this study. Only minor variations are observed in both the virus and the serum titers.

3. The homogeneity of successive virus strains as determined by heterologous antibody

Herpes virus strains A and B, isolated successively from subject G. G., were tested further by cross-neutralization with specific rabbit antiserums (anti-A, anti-B) for evidence of antigenic dissimilarity. From table 4 it is seen that there was no indication of antigenic difference in the two strains.

TABLE 4.—*The cross-neutralization in mice of herpes simplex virus strains A and B with strain-specific rabbit antiserums.*

Herpes simplex virus strain	Negative log of LD_{50}/0.03 ml	PD_{50} titers* of serum samples	
		Anti-A	Anti-B
A	3.0	12.5	15
B	3.0	11	15.8

* The identity in the LD_{50} titers of the strains of herpes simplex virus made it unnecessary to employ the factor 6^n to correct the PD_{50} titers.

DISCUSSION

A means of correcting antibody titers for variation in the potency of test virus preparations greatly facilitates the comparison of neutralization tests. The observed definition, within limits, of a quantitative relationship between virus titer and antiserum concentration for 50% neutralization is in general accord with similar findings of Horsfall[2] and Kilbourne and Horsfall[3] for influenza virus, and of Morgan[4] for equine encephalomyelitis virus. Jawetz and Coleman,[5] however, in dealing with neutralization tests in ovo found that undiluted antiserum neutralized disproportionately more herpes simplex virus than diluted antiserum.

2. Horsfall, F. L., Jr. 1939, Neutralization of epidemic influenza virus; linear relationship between quantity of serum and quantity of virus neutralized. J. Exper. Med. **70:** 209–222.
3. Kilbourne, E. D. and Horsfall, F. L., Jr. 1951, Mouse-egg neutralization. Neutralization in the mouse of influenza viruses not adapted to the mouse. J. Immunol. **67:** 431–436.
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5. Jawetz, E. and Coleman, V. R. 1952, Studies on herpes simplex virus. III. The neutralization of egg-adapted herpes virus by human sera *in ovo*. J. Immunol. **68:** 645–653.

TABLE 3.—*The cross-neutralization in mice of successive isolates from a human subject of herpes simplex virus (strains A-D) with serum samples obtained concomitantly (samples a-d) and 3 months after the last virus isolation (sample e).*

Herpes simplex strain	Negative log of LD_{50}/0.03 ml	PD_{50} titers of serum samples					$PD_{50}/6^n$ titers of serum samples				
		a	*b*	*c*	*d*	*e*	*a*	*b*	*c*	*d*	*e*
A	3.0	16	18.6	15.8	18.6	18.6	8.3	9.7	8.2	9.7	9.7
B	3.0	19.5	15.8	15.8	15.8	12	10	8.2	8.2	8.2	7.2
C	3.5	8	8	11	11		8*	8*	11^4	11*	
D	2.5	46	64	46	46		7.7	10.7	8.3	7.7	

* Correction with 6^n factor not applied. Interpolation was made on the basis of log LD_{50} 3.5.

The factors responsible for the immunological pattern of herpes simplex infections may include the level of circulating antibody, local resistance, individual susceptibility, virulence, and antigenic differences between virus strains. At any one time one or more of these factors may decide the outcome of the host-parasite relationship. Although limited strain differences may exist among strains of virus passed in animals or among strains isolated from different anatomical sites of different individuals,[6–8] there seems to be little factual support for the assumption that such antigenic differences are responsible for the recurrence of herpetic lesions. The results of the study reported here provide no evidence to the contrary. Furthermore, the hypothesis presented by Brain[9] in 1937, and more recently by Jawetz et al,[10] that a fall in the serum antibody titer of a herpetic individual may be associated with the appearance of a herpetic eruption finds no support in our experiments. Indeed, it was observed that vesicles did recur despite circulating antibody in high titer. It appeared that endogenous virus isolated from a single individual at different intervals of time during the course of recurrent herpetic attacks had not altered in potency, and that his circulating antibody titer remained relatively constant even 3 months after the appearance of the last vesicle. While it may be reasonable to assume that neutralizing antibodies in the circulation are capable of focalizing virus upon recurrent infection, the importance of antibody in preventing the appearance of recurrent herpetic lesions may be overestimated.

SUMMARY

Four strains of herpes simplex virus were isolated from a single human subject upon four successive recurrent attacks of herpes simplex. These four strains upon antigenic analysis by cross-neutralization tests with homologous and heterologous antibody yielded no evidence for antigenic dissimilarity. Moreover, the presence of high level neutralizing antibodies in the blood of the individual did not prevent the appearance of recurrent herpetic lesions.

Evidence was found in support of a linear relationship between the concentration of herpes simplex virus and the PD_{50} titers of the antiserums used in successive neutralization tests in mice. The recognition that within certain limits a 10-fold increase in the concentration of the virus corresponded to a 6-fold increase in the PD_{50} titer of serum, resulted in the acceptance of this ratio for the interpolation of the findings in cross-neutralization tests. This simple method made easy the interpretation of the results.

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STUDIES ON HERPES SIMPLEX VIRUS

II. AN AFFINITY IN VITRO OF HERPES VIRUS FOR RABBIT MICROPHAGES AND MACROPHAGES

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The role of phagocytosis in resistance to recurrent herpetic infection is little understood. In 1934, Jamuni and Holden[1] reported that either normal rabbit leucocytes, or leucocytes from herpes-immune rabbits, enabled antiherpetic rabbit serum to nullify the pathogenic effects of herpes virus under circumstances where the antibody content of the antiserum was inadequate to neutralize the quantity of herpes virus employed. Neutralization of herpes virus also was obtained when leucocytes from immune animals were employed in combination with normal serum. However, normal leucocytes in the presence of normal serum were ineffective. These observations were accentuated when mononuclear cells were used. The age of the host has been shown to influence experimental herpetic infections. For example, Lennette and Koprowski,[2] in 1944, indicated that young mice, upon infection by an extraneural route were more susceptible to herpes simplex virus than older mice. Moreover, it has been demonstrated that the virus of herpes simplex, when introduced into young mice, is disseminated in the animal body both by neural and extraneural routes.[3–6]

The purpose of the experiments reported herein was to study the affinity of herpes simplex virus for rabbit microphage and macrophage cells (MM cells) in an attempt to explore the role of phagocytosis in recurrent herpetic infections.

MATERIALS AND METHODS

Source of MM cells.—A New Zealand white rabbit was inoculated intraperitoneally with 800 ml of sterile 0.85% sodium chloride. Eighteen hours later, 25 ml of peritoneal fluid were aspirated with an 18 gauge needle into a 50 ml syringe. The fluid was centrifuged without delay

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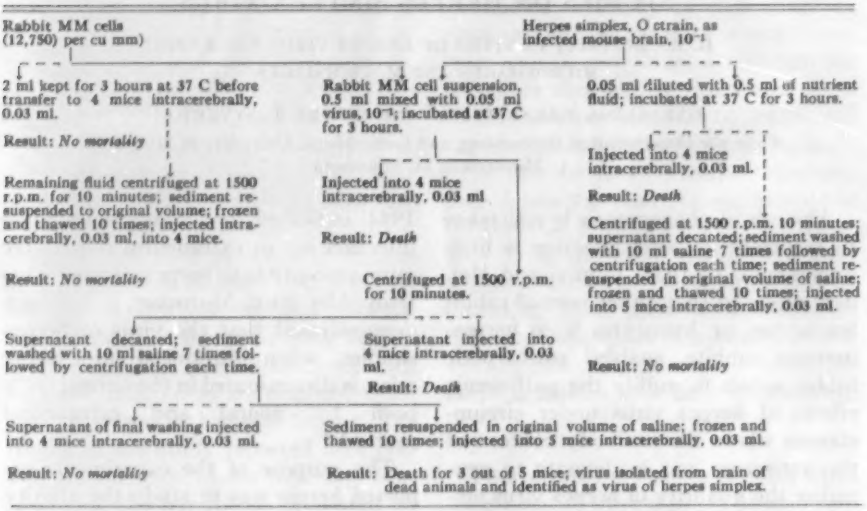
TABLE 1.—*The affinity of herpes virus for rabbit microphages and macrophages in vitro.*


Rabbit MM cells (12,750) per cu mm)
Herpes simplex, O strain, as infected mouse brain, 10⁻¹
2 ml kept for 3 hours at 37 C before transfer to 4 mice intracerebrally, 0.03 ml.
Result: No mortality
Remaining fluid centrifuged at 1500 r.p.m. for 10 minutes; sediment resuspended to original volume; frozen and thawed 10 times; injected intracerebrally, 0.03 ml, into 4 mice.
Result: No mortality
Rabbit MM cell suspension, 0.5 ml mixed with 0.05 ml virus, 10⁻¹; incubated at 37 C for 3 hours.
Injected into 4 mice intracerebrally, 0.03 ml
Result: Death
Centrifuged at 1500 r.p.m. for 10 minutes.
Supernatant decanted; sediment washed with 10 ml saline 7 times followed by centrifugation each time.
Supernatant of final washing injected into 4 mice intracerebrally, 0.03 ml.
Result: No mortality
Supernatant injected into 4 mice intracerebrally, 0.03 ml.
Result: Death
Sediment resuspended in original volume of saline; frozen and thawed 10 times; injected into 5 mice intracerebrally, 0.03 ml.
Result: Death for 3 out of 5 mice; virus isolated from brain of dead animals and identified as virus of herpes simplex.
0.05 ml diluted with 0.5 ml of nutrient fluid; incubated at 37 C for 3 hours.
Injected into 4 mice intracerebrally, 0.03 ml.
Result: Death
Centrifuged at 1500 r.p.m. 10 minutes; supernatant decanted; sediment washed with 10 ml saline 7 times followed by centrifugation each time; sediment resuspended in original volume of saline; frozen and thawed 10 times; injected into 5 mice intracerebrally, 0.03 ml.
Result: No mortality

for 5 minutes at 1000 r.p.m. and the sediment resuspended in 10 ml of a medium made from 40 parts Hanks's balanced salt solution, 40 parts guinea pig serum and 20 parts chick embryo extract.

Cell count.—The total number of cells in the suspension were enumerated by employing a whiteblood cell counting pipette and a Neubauer chamber. The average of 5 counts of the suspension was 12,750 cells per cu mm. Smears stained by Wright's method showed polymorphonuclear leucocytes and macrophages in approximately equal numbers.

Phagocytic activity of MM cells.—A hanging drop preparation was made with a mixture of one drop of MM cells in suspension and a drop of India ink, 1:160, in water. The slide was placed in an incubator for 3 hours at 37 C, after which it was examined microscopically. From 25 to 30% of the cells were heavily loaded with carbon particles.

Virus.—Mouse brain infected with the O strain of herpes simplex virus was used in 10% suspension for these experiments. This strain of herpes simplex had been isolated by one of the authors (J.T.S.), in 1946, from a patient with aphthous stomatitis, conjunctivitis, and balanoposthitis. It was in its 13th mouse brain passage with a negative log of 4.8 for the LD_{50}, as determined in 8- to 9-week-old albino mice (CFW), by the intracerebral injection of 0.03 ml.

RESULTS AND DISCUSSION

The protocol that was followed and the results that were obtained are shown in table 1.

The findings indicate (a) that the phagocytic cells, whether intact or disrupted as a result of repeated freezing and thawing, were not lethal for mice by intraperitoneal injection (column to left), (b) that the virus of herpes simplex had been removed from the test infected mouse brain suspension by washing 7 times with physiological saline followed by freezing and thawing 10 successive times, as shown by the noninfectiousness for laboratory mice of the suspension after this treatment (column to right), and, finally, (c) that the virus of herpes simplex had been phagocytosed by the MM cells, or firmly attached to them (middle column). The conclusion that virus must have been phagocytosed resulted when it was learned that the final supernatant fluid derived from the mixture of MM cells and herpes simplex virus upon test in mice was innocuous,

in contrast to the sediment which resulted for 3 of 5 mice in death from herpetic encephalitis. This evidence was supported by the failure to demonstrate virus after ten successive attempts were made to elute it from these phagocytic cells. The reason for the greater susceptibility of young mice to infection with the virus of herpes simplex upon intraperitoneal injection[2] is not known. It may result (a) from the inadequate or immature phagocytic response of younger animals and/or (b) from the naturally acquired resistance (specific or nonspecific) to infection by the virus of herpes simplex that occurs with increasing age. It is significant that both age groups of mice were equally susceptible to infection when the intracerebral route of inoculation was used for testing the virus.[7]

The effect of cortisone and gastric mucin upon the infectivity of herpes simplex virus in laboratory mice is reported in an accompanying paper.[7] Cortisone may have an inhibitory effect on cellular reaction and/or a depressing effect on the breakdown of virus in phagocytic cells.[8,9] Consequently, the increase in susceptibility of adult mice under the influence of cortisone to the intraperitoneal injection of herpes simplex virus may have resulted from either or both of these effects of cortisone.[7]

The results of the present study show that phagocytic cells readily acquire herpes virus and permit its survival for hours. This demonstration of an affinity of herpes virus for phagocytic cells may reflect active phagocytosis or adsorption to the cells. Comparable studies of the effect of immune serum on the rate of virus uptake were not carried out. Despite these omissions in control studies, the findings were interpreted tentatively as evidence for phagocytosis. In support of this tentative interpretation was our failure to elute virus from phagocytic cells by washing repeatedly and our successful demonstration of infectious virus in cells which had been disrupted by freezing and thawing ten times. It is assumed from this suggestive evidence for phagocytosis in experimental herpetic infection that phagocytic cells by active phagocytosis may play a role in the dissemination or destruction of the virus of herpes simplex. This possibility leaves open for patients subject to recurrent attacks of herpes simplex, the relationship (a) of naturally acquired host resistance to the phagocytic activity of cells, and (b) of reinfection to the residence of virus locally in macrophages and its release by any untoward circumstance that results in injury to the macrophage.

If phagocytes can ingest herpes virus, as is suggested by our experiments, then phagocytosis may play a role in the dissemination or destruction of the virus of herpes simplex. Determining factors in the recurrence of herpetic infection then could include (a) the relationship of naturally acquired host resistance to the activity of phagocytic cells, and (b) the local residence of virus in macrophages and its release by any injury to them.

SUMMARY

The results of in vitro experiments in which rabbit microphage and macro-

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phage cells and herpes simplex virus as 10 per cent infected mouse brain suspension were mixed indicated that the virus was either phagocytosed by the phagocytic cells or became firmly attached to them. Despite the absence of irrefutable evidence for phagocytosis, it is suggested that phagocytic cells may play an important role in disseminating and/or destroying the virus of herpes simplex. The rate of virus destruction in phagocytic cells may depend upon the relative naturally acquired resistance of the host.

STUDIES ON HERPES SIMPLEX VIRUS

III. THE EFFECTS OF ROENTGEN RADIATION, CORTISONE AND GASTRIC MUCIN UPON THE INFECTIVITY OF HERPES SIMPLEX VIRUS FOR LABORATORY MICE

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It has seemed reasonable to assume that the pathogenesis of recurrent herpetic infection is determined by an alteration in host resistance. The immunological investigation reported previously[1] provided no evidence that fluctuations in the titer of autogenous virus or of the antibody circulating in its host influenced the course of infection. Hence, it was of interest to inquire whether herpes simplex virus infection was susceptible to the action of agents capable of altering the natural resistance of the host.

The present study was undertaken to learn the effects (a) of X ray, cortisone, and mucin, singly or in combination, upon infections induced by the virus of herpes simplex in 8- to 9-week-old Swiss albino mice and (b) of cortisone and mucin upon the infectivity for mice of the same virus when administered by different routes and to assorted age groups.

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1. Garabedian, G. A. and Syverton, J. T. 1953, Studies on herpes simplex virus. I. An antigenic analysis of four strains of virus isolated from a human subject. J. Infect. Dis. **95**: 1–4.

MATERIALS AND METHODS

Method of radiation.—Radiation generated by a current of 15 milliamperes with a 250 kv peak was administered in a single massive dose to the whole body at a target skin distance of 60 cm, in a 14 cm field. Filters of 0.5 mm of copper and 1.0 mm of aluminum (HVL 1.35 mm Cu) were employed. The radiation output, as determined by a Victoreen "r"-meter, was 41.5 r per minute.[2]

Cortisone.‡—Cortisone acetate (Cortone, Merck) was diluted with sterile physiological saline to a final concentration of 20 mg per ml.

Mucin.§—A 5% suspension of granular mucin in distilled water was prepared in a single lot by trituration, autoclaved at 115 C for 20 minutes, and adjusted to pH 7.3 with sterile $N/20$ sodium hydroxide.

Virus.—The strain O herpes simplex virus used in these studies was isolated by one of us (J.T.S.) in 1946 from a patient with aphthous stomatitis, conjunctivitis and balanoposthitis. The virus was stored as lyophilized 20% mouse brain suspension; in its 13th passage an LD_{50} of -4.8 was observed, as determined by the intracerebral injection of 0.03 ml into 8- to 9-week-old Swiss albino mice (CFW strain).

EXPERIMENTS

1. The effect of agents capable of modifying host resistance on herpes simplex infection in mice

The influences of roentgen radiation, cortisone and gastric mucin on herpes

2. Syverton, J. T., Werder, A. A., Friedman, J., Graham, A. B. and Mira, O. J. 1952, Cortisone and roentgen radiation in combination as synergistic agents for production of lethal infections. Proc. Soc. Exper. Biol. & Med. **80**: 123–128.

‡ Cortisone acetate (Cortone), Merck and Company, Rahway, New Jersey.

§ Granular mucin type 1701-W.

TABLE 1.—*A summary of experimental procedures for testing the effects of roentgen radiation, cortisone and mucin upon the infectivity of herpes simplex virus for 8- to 9-week-old Swiss albino mice.*

Host mice		Treatment				
Group (64 each)	Lot (16 each)	X radiation 200r/mouse	Cortisone acetate 2 mg/0.1 ml	Mucin, 5%, and O strain, $10^{-1.3}$, 0.2 ml	Broth and O strain, $10^{-1.3}$, 0.2 ml	Mucin, 5%, and noninfected mouse brain, $10^{-1.3}$, 0.2 ml
B_1	A_1	Yes	No	Yes	No	No
	A_5	Yes	Yes	Yes	No	No
	A_9	No	Yes	Yes	No	No
	A_{13}	No	No	Yes	No	No
B_2	A_2	Yes	No	No	Yes	No
	A_6	Yes	Yes	No	Yes	No
	A_{10}	No	Yes	No	Yes	No
	A_{14}	No	No	No	Yes	No
B_3	A_3	Yes	No	No	No	Yes
	A_7	Yes	Yes	No	No	Yes
	A_{11}	No	Yes	No	No	Yes
	A_{15}	No	No	No	No	Yes
B_4	A_4	Yes	No	No	No	No
	A_8	Yes	Yes	No	No	No
	A_{12}	No	Yes	No	No	No
	A_{16}	No	No	No	No	No

virus infection were tested in 8- to 9-week-old Swiss albino mice, almost exclusively males from 20 to 25 g in weight. As indicated in table 1, 16 lots of 16 mice were employed, of which 8 lots were irradiated 24 hours prior to inoculation, 4 of the irradiated lots and 4 other lots received 2 mg doses of cortisone subcutaneously on the following day, and the 4 remaining lots were not treated. Before inoculation the 256 animals were regrouped into 4 sets, each consisting of 3 subsets of prepared animals and one control subset. Two major sets were injected intraperitoneally with strain O virus as a $10^{-1.3}$ mouse brain suspension, mixed with either 5% mucin or nutrient broth. A third major set was inoculated with $10^{-1.3}$ noninfected mouse brain plus mucin, while the fourth was not inoculated.

The findings of the experiment are summarized in figure 1. It can be seen that for either cortisone- or radiation-treated animals the mixture of mucin with virus injected intraperitoneally increased the mortality (compare Group B1/A9 with B2/A10, B1/A1 with B2/A2). This effect of mucin was diminished when both irradiation and cortisone treatment were employed (compare B1/A5 with B2/A6), and when all pretreatment was omitted (compare B2/A14 with B2/A4). For mice injected with virus and broth the effect of radiation treatment on virus-induced mortality was small (compare B2/A2 with B2/A4), an enhancive effect of cortisone treatment was observed (compare B2/A10 with B2/A4), while the combined effect (B2/A6) was greater than the sum of the separate effects. The effect of herpes simplex virus as a lethal agent was reduced progressively when the virus was injected in mixture (a) with mucin into irradiated mice, (b) with broth into cortisone treated animals, (c) with mucin into untreated mice, (d) with broth into irradiated mice, and (e) with broth into untreated mice.

2. The influence of host age and inoculation route on the effects of cortisone and gastric mucin on herpes simplex infection of mice

Strain O herpes simplex virus, in combination respectively with broth, mucin, or mucin and cortisone (tables 2 and 3) was injected either intracranially or intraperitoneally into 3 or 8 week old mice. Four Swiss albino mice (CFW strain) were inoculated with each broth

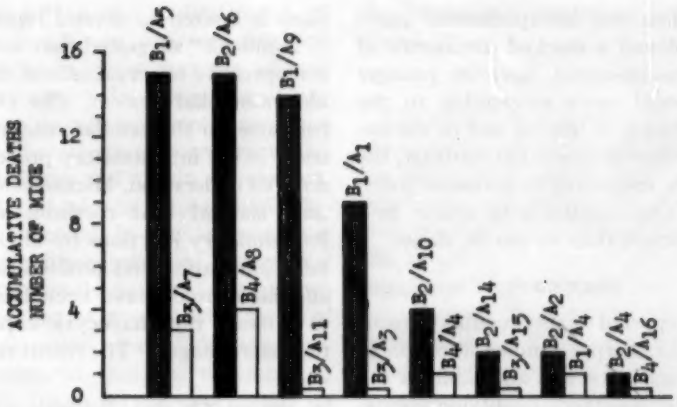

FIG. 1.—The effects of Roentgen radiation, cortisone and gastric mucin, singly or in combination, upon infections induced by the virus of herpes simplex in 8- to 9-week-old Swiss albino mice.

Key (see table 1)

B_1/A_5. — Virus + mucin + x-radiation + cortisone
B_2/A_7. — ——— + mucin + x-radiation + cortisone
B_2/A_8. — Virus + broth + x-radiation + cortisone
B_4/A_8. — ——————— + x-radiation + cortisone
B_1/A_9. — Virus + mucin + cortisone
B_2/A_{11}. — ——— + mucin + cortisone
B_1/A_1. — Virus + mucin + x-radiation
B_2/A_3. — ——— + mucin + x-radiation
B_2/A_{10}. — Virus + broth + cortisone
B_4/A_4. — ——————— + cortisone
B_2/A_{14}. — Virus + mucin
B_3/A_{15}. — ——— + mucin
B_2/A_2. — Virus + broth + x-radiation
B_1/A_4. — ——————— + x-radiation
B_2/A_4. — Virus + broth
B_4/A_{16}. — Not inoculated

dilution of virus, as infected mouse brain suspension, in amounts of 0.03 ml for the intracranial route and 0.2 ml for the intraperitoneal route. Cortisone acetate in sterile physiological saline was administered, where indicated, in 0.1 ml amounts (2 mg) 2 hours prior to the injection of virus.

The importance of the route of inoculation to the problem under study is brought out by the results given in tables 2 and 3. The infectivity of herpes simplex virus injected intracranially was not influenced by mucin, treatment of the host with cortisone, or host age, within the limits of study. On the other

TABLE 2.—*The comparative effects of mucin and cortisone, singly and in combination, upon herpes simplex infection, O strain, in 3- and 8-week-old laboratory mice inoculated intracranially.*

Age of mice in weeks	Preparation of animals: Groups	Preparation of animals: Cortisone	O strain of herpes simplex virus in admixture with:	Results: Negative log of LD_{50}/0.03 ml
3	A	No	Mucin	4.5
	B	Yes	Mucin	4.9
	C	No	Nutrient broth	4.7
8	A	No	Mucin	4.4
	B	Yes	Mucin	4.4
	C	No	Nutrient broth	4.5

TABLE 3.—*The comparative effects of mucin and cortisone, singly or in combination, upon herpes simplex infection, O strain, in 3- and 6-week-old mice inoculated intraperitoneally.*

Age of mice in weeks	Preparation of animals: Groups	Preparation of animals: Cortisone	O strain of herpes simplex virus in admixture with:	Results: Negative log of LD_{50}/0.03 ml
3	A	No	Mucin	5.0
	B	Yes	Mucin	5.0
	C	No	Nutrient broth	3.9
6	A	No	Mucin	1.9
	B	Yes	Mucin	2.8
	C	No	Nutrient broth	1.3

hand, when the intraperitoneal route was employed a marked divergence of results was observed. Here the younger mice proved more susceptible to the virus in broth or mucin, and to the enhancive effect of mucin; in contrast, the older mice responded to cortisone treatment in combination with mucin to a greater extent than to mucin alone.

DISCUSSION

The reported experiments demonstrated that herpes simplex infection in Swiss albino mice was amenable to the influence of resistance-modifying agents. It was observed that this influence was manifest when mice were infected by the intraperitoneal route. When the agents were tested singly only cortisone was particularly effective; considerable modifications of host resistance were obtained with combinations of mucin and cortisone treatment, mucin and X radiation, or cortisone treatment and X radiation. The last combination was most active, to a degree exceeding that expected from summation. This finding confirms and extends previous studies, which showed that cortisone and roentgen radiation in combination enhanced synergistically infections induced in mice by poliomyelitis and Coxsackie viruses, *Candida albicans* and *Blastomyces dermatitidis*.[2] Although mucin has been used as an enhancive agent for a variety of bacterial infections in laboratory animals,[3–9] its use for viral infections is limited to several reports.[10–12]

Tunnicliff[13] suggested that mucin has a suppressive effect on cells of the reticulo-endothelial system. The effects of cortisone on the cellular reactions that occur in the inflammatory processes are not well understood. Michael and Whorton[14] showed that cortisone modified inflammatory reactions by altering cellular infiltration and proliferation. The adrenal steroids have been found also to activate the phagocytic capacity of the macrophages.[15] The recent report by

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Lurie et al[16] on the multiplication of tubercle bacilli in macrophages led Fagraeus[17] to suggest that the adrenal hormones act by depressing the ability of phagocytic cells to destroy infectious agents. The effects of roentgen radiation, cortisone and mucin on the susceptibility of mice to herpes simplex infection observed in our experiments, and the nullifying effect of intracranial inoculation on the action of the last two agents, might be interpreted in terms of the modification of a cellular response. It is suggested that the difference in susceptibility to herpes virus shown by the older groups of mice upon treatment with mucin, or cortisone and mucin, as compared with no treatment, may have resulted from (a) the inhibitory effect of cortisone and mucin on cellular reaction and/or (b) the depressive effects of the agents on the destruction of the virus in phagocytic cells. The influence of cortisone is of particular interest in indicating a possible role of hormone action in the pathogenesis of herpetic recurrences.

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SUMMARY

The effects were studied (a) of roentgen radiation, cortisone and gastric mucin, singly or in combination, upon the infectivity of herpes simplex virus for 8- to 9-week-old Swiss albino mice, and (b) of cortisone and mucin upon the infectivity of the same virus when administered to Swiss albino mice by different routes and to various age groups. Mucin in mixture with virus by injection intraperitoneally increased mortality among cortisone-treated or irradiated animals. The enhancive effect of mucin was decreased when treatment with cortisone and radiation was either combined or omitted. Radiation had little effect on the mortality from virus injected in broth, cortisone treatment increased mortality, and combined treatment resulted in a synergistic enhancement. The lethality of herpes virus inoculated intracerebrally was not affected by mucin, cortisone treatment of the host, or host age. Younger mice when inoculated intraperitoneally were more susceptible to the virus in broth or mucin, and mucin exerted an enhancive effect; older mice infected by this route were more responsive to the combined effects of mucin and cortisone treatment.

FURTHER STUDIES OF GROUP A STREPTOCOCCAL FACTORS WITH LETHAL AND CARDIOTOXIC PROPERTIES

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In a previous communication (Schwab, Watson and Cromartie, 1953) it was reported that a reaction resembling the generalized Shwartzman phenomenon could be produced with soluble products of group A streptococci. American Dutch rabbits were injected intravenously with an extract of a streptococcal skin lesion, followed by a second intravenous injection of either a filtrate of a culture of *Salmonella typhosa* or a reduced culture filtrate of streptococci which had a high titer of streptolysin O. The prominent features of the reaction are death and necrosis of cardiac muscle. The heart lesions resemble those described as myofiber necrosis by Thomas, Denny, and Floyd (1952a, 1952b, 1953) and also present some features of the lesions reported by Murphy and Swift (1950).

In the previous report (Schwab et al, 1953) it was suggested that streptolysin O was the factor responsible for the provoking activity of the reduced streptococcal culture filtrate. It is the purpose of this paper to record further studies which indicate that this assumption was correct. Studies are also presented on the nature of the active material present in the lesion extract which potentiates the toxic action of streptolysin O.

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MATERIALS AND METHODS

Streptococcal skin lesion extract.—The preparation of this extract was described previously (Schwab et al, 1953). Briefly, the technique consists of intracutaneous injection of 30 ml of a broth culture of group A streptococci in about 60 sites over the shaved abdomen and thorax of 2 kg New Zealand white rabbits. The moribund animals are killed after 18 hours, the lesion ground in a meat grinder and extracted overnight with an equal volume of 0.85% sodium chloride solution at 2 C. The extract is then centrifuged at 25,000 r.p.m. in the Spinco 30 head for ½ hour. The supernatant is filtered through a Selas bacterial filter and frozen at −70 C. This contains the preparing factor. Usually 0.5 ml is sufficient for a preparative dose.

Reduced culture filtrates of group A streptococci.—A 16-hour Todd-Hewitt broth culture of the streptococcus was filtered, reduced, and stored as described by Todd (1938). It was titered for streptolysin O activity using standard antistreptolysin O. Three preparations were used in the work reported in this paper. Two were prepared with a type 3 (D-58) strain, and both had titers of 12.5 combining units per ml. A provoking dose of the first lot was 2.0 ml and of the second lot, 4.0 ml. A type 12 (Rammelkamp) strain was used for the third preparation; the titer was 6 combining units per ml and a provoking dose was 4.0 ml.

Preparation and provocation of the reaction.—American Dutch rabbits weighing about 1 kg were used. The preparative intravenous injection consisted of 0.25 to 0.5 ml of the lesion extract, depending upon the lot of material used. The intravenous provocative injection was given after an interval of 4 to 16 hours. This consisted of 2.0 to 4.0 ml of the reduced streptococcal culture filtrate. The volume used varied with the activity of the preparation.

Bacterial toxins.—The filtrate of a broth culture of a N-28-1 strain of *S. typhosa* is referred to in this paper as "typhoid culture filtrate." The details of its preparation and determination of its potency were given previously (Schwab et al, 1953).

EXPERIMENTAL

Identification of the provoking factor in the reduced streptococcal culture filtrate.—Table 1 summarizes the results thus far of rabbits prepared with the lesion extract and provoked with the reduced streptococcal culture filtrate. It may be seen that 47% died. The survivors were killed 48 to 72 hours after the provocative injection. Histologic studies were made on the hearts of all animals sacrificed and on 5 of those which died as a result of the reaction. Lesions previously described as myofiber necrosis were found in 24 of these 43 animals. Out of a total of 82 rabbits, 58 either died or displayed cardiac lesions, giving a result of 70% positive. Neither death nor development of lesions was observed in control animals given only a single injection of the reduced streptococcal culture filtrate or those prepared with lesion extract and provoked with reduced sterile broth.

Experiments of an indirect nature all indicate that the provoking activity of the reduced culture filtrate is associated with the streptolysin O molecule. Each of the following groups of rabbits was prepared with the lesion extract, and after an interval of 4 hours was given the reduced filtrate subjected to various procedures known to inhibit the activity of streptolysin O. The filtrate used in these experiments contained 12.5 combining units (Todd, 1938) of streptolysin O per ml, and a provoking dose was 4.0 ml.

One experiment of this nature involved the inhibition of streptolysin O with rabbit antiserum against streptococcal filtrates. While this antiserum had a high antistreptolysin O titer, it also contained antibodies against many of the other antigenic products of streptococci. Each rabbit received 4.0 ml of the reduced filtrate, containing 50 combining units of streptolysin O, which had been incubated at 25 C for ½ hour with 60 antistreptolysin units. In a second experiment, rabbits were provoked with the reduced filtrate after the lytic activity had been removed by the addition of cholesterol in a final concentration of 1 to 200,000. The cholesterol suspension was prepared according to the technique of Cohen et al (1937). Five mg of cholesterol was dissolved in 5.0 ml of absolute methanol. This was poured slowly into 100 ml of boiling water and filtered. A volume of 2.4 ml of this suspension was added to 24 ml of the reduced broth filtrate and the mixture kept at room temperature for ½ hour. Each rabbit received 4.4 ml as a provoking dose. There is, of course, no evidence that cholesterol could not inhibit the activity of other streptococcal products. A third experiment involved the inactivation of streptolysin O by bubbling oxygen through the solution. A period of 2 hours at room temperature was required to inactivate the lytic activity. The acid produced as a result of oxidation of the reducing agent was neutralized periodically with 0.1 *N* sodium hy-

TABLE 1.—*Reaction in rabbits prepared with lesion extract and provoked with reduced streptococcal culture filtrate.*

Procedure	Death		Microscopic cardiac lesions	
	Total	Dead	Total studied	Number positive
Prepared I.V. with lesion extract, provoked after 4 hours with reduced streptococcal culture filtrate	82	39	43*	24
Single injection of reduced streptococcal culture filtrate	35	0	15	0
Prepared I.V. with lesion extract and provoked after 4 hours with reduced sterile broth	14	0	14	0

* 38 of the animals studied were sacrificed.

TABLE 2.—*Effect of streptolysin O inhibitors on provoking activity of reduced filtrates.*

Provoking material*	Number of rabbits	Number dead	Number of cardiac lesions	Total either dead or lesions
Reduced streptococcal culture filtrate	11	6	3	9
Reduced filtrate + antistreptolysin O	11	0	0	0
Reduced filtrate + cholesterol	11	0	1	1
Reduced filtrate oxidized with oxygen	10	0	3	3
Reduced filtrate of Blackwell strain	5	0	0	0
Reduced filtrate only	6	0	0	0

* Rabbits were given a preparative injection of the lesion extract 4 hours previously.

droxide. Other streptococcal enzymes such as proteinase, however, are also known to be active only in the reduced state. Each rabbit was injected with 4.0 ml of this oxidized filtrate.

A fourth group of rabbits received a provoking injection of 4.0 ml of a reduced filtrate of a Todd-Hewitt broth culture of a group A, type 11, Blackwell strain of streptococci, which produces no streptolysin O. It is probable that this filtrate contained most of the other metabolites of group A streptococci.

A fifth group, serving as a control, received as a provoking dose 4.0 ml of the unaltered reduced filtrate which had been left exposed to the air for ½ hour at 25 C. Another control group received only the injection of the unaltered reduced culture filtrate. The criteria of positive reactions were death of the animals or the demonstration of cardiac lesions in the survivors. The nature of the cardiac lesions was previously described (Schwab et al, 1953).

This series of experiments was repeated twice and the results are presented in table 2. It is apparent that in each case in which the streptolysin O had been inactivated before injection, the incidence of death and the occurrence of cardiac lesions was absent or greatly reduced in comparison with the control group which had received the unaltered reduced culture filtrate. The cardiac lesions in 3 rabbits in the group provoked with the oxygenated streptolysin O might be a result of the possibile in vivo reversibility of this reaction, in contrast to the inhibition of the enzyme with antiserum or cholesterol which is not readily reversible.

The above provoking materials were checked before injection to ensure that no streptolysin O lytic activity remained after incubation with the inhibiting agents. This was done by adding an aliquot of each mixture, before and after incubation with the respective streptolysin inhibitor, to a 5% suspension of washed rabbit erythrocytes. After incubation at 37 C for 1 hour no lytic activity could be detected in any of the solutions to be injected, other than the unaltered control. Each produced rapid lysis of the red cells before the addition of the inhibitor.

Nature of the preparing factor in the streptococcal skin lesion extract.—To establish whether the production of the preparing factor is confined to certain strains of streptococci or is a property common to all group A streptococci, lesion extracts were prepared with various group A strains and the presence of the preparing factor determined. The extracts were prepared under similar conditions, using the following strains of group A organisms; type 4—366, type 4—T4, type 12—B-25, and type 28. In these experiments death of the animal was used as the criterion of a positive reaction. Comparative titrations are shown in table 3. The types 28, 12, and 366 strain of type 4 were all comparable in the production of the preparing factor. The lesion extract produced with the T4 strain did not possess

TABLE 3.—*Preparative factor from various strains of group A streptococci.*

Strains used to produce preparing factor	Number of rabbits	Number dead
Type 28	5	4
Type 12 (B-25)	5	3
Type 4 (366)	5	3
Type 4 (T 4)	5	0

All animals provoked after 4 hours with streptolysin O.

any of the preparing factor, even when the volume of the lesion extract was varied from 0.25 ml to 1.0 ml.

The effect of heat on the preparing factor present in the lesion extracts was determined by heating the extract at 60 C for ½ hour. The results of two such experiments shown in table 4 indicate that the preparative factor is destroyed by the treatment.

The following experiment was carried out to determine if the preparing factor present in the lesion extracts is retained within a Visking dialyzing membrane after dialysis for 24 hours against distilled water. A euglobulin fraction which precipitated during the dialyzing procedure was resuspended in saline to about 1.0% protein as determined by nitrogen analysis. The supernatant was lyophilized to a dry powder and dissolved in saline to about 1.0% concentration by weight. Rabbits were prepared with 0.5 ml of each of these fractions and provoked with "typhoid culture filtrate." Table 5 shows that both fractions possessed good preparing activity, which suggests that the preparing factor is retained by the Visking dialyzing membrane.

TABLE 4.—*Effect of heat on preparative factor.*

Treatment of preparing factor	Exp. no.*	Number of rabbits	Number dead
Heated 60 C ½ hour	1	4	0
	2	6	0
Controls—not heated	1	4	2
	2	6	4

* Exp. 1.—Rabbits provoked after 4 hours with streptolysin O.
Exp. 2.—Rabbits provoked after 4 hours with "typhoid culture filtrate."

DISCUSSION

In a recent review of the pathogenic properties of group A streptococci, it was pointed out that present knowledge does not permit an understanding of the mechanisms whereby these organisms bring about a wide variety of tissue changes (Watson and Cromartie, 1951). Aside from the rather clear relations between the skin lesion of scarlet fever and erythrogenic toxin little information is available concerning the manner in which these organisms damage tissue and produce death.

Recently reported observations (Schwab, Watson and Cromartie, 1953) revealed that when group A streptococci multiply in the skin of rabbits a material is produced which is capable of markedly altering the reaction of American Dutch rabbits to a subsequent injection of a reduced broth culture filtrate of group A streptoccocci. The experiments reported here confirm the belief that streptolysin O is the active provoking factor in the reduced filtrate. As pointed out above, each of these experiments is of an indirect nature involving the inhibition of the lytic activity of the filtrate. None of the methods used is specific for streptolysin O, but until a purified product is available these techniques must suffice. These criteria have been used to identify streptolysin O in previous studies (Bernheimer, 1948).

Some of the properties of the factor produced in the streptococcal lesion which potentiates the activity of strepto-

TABLE 5.—*Effect of dialysis and lyophilization on preparative factor.**

Treatment of preparing factor	Number of rabbits	Number dead
Dialyzed, lyophilized, dissolved to give 1.0% solution	8	6
Water insoluble fraction dissolved in saline to give 1.0% solution	6	4
Single injection of "typhoid culture filtrate"	6	0

* All animals were provoked after an interval of 16 hours with "typhoid culture filtrate."

lysin O are also demonstrated; this substance is produced by many but not all strains of group A streptococci. The active material is heat labile, withstands lyophilization and may be of large molecular size since it will not pass through a Visking dialyzing membrane.

Further studies will be required to determine if the mechanism involving the combined action of the two soluble products of group A streptococci under study plays any role in the pathogenesis of natural infections with these organisms. However, these studies suggest another approach that might be used in evaluating the lethal and tissue-damaging properties of other streptococcal products. This method of study might demonstrate toxic properties not obvious when other methods of testing are utilized.

The technique used to demonstrate the effects of the combined action of streptolysin O and the material extracted from the skin lesions is similar to that utilized to bring about the generalized Shwartzman reaction. Until more precise information is available concerning the mechanisms responsible for the lethal effect and tissue damage associated with these reactions, it will be fruitless to speculate whether or not the mechanisms are the same.

SUMMARY

Further studies of a mechanism that seems related to the lethal and tissue damaging property of group A streptococci are reported. When group A streptococci multiply in the skin of rabbits a material is produced which is capable of markedly altering the reactions of American Dutch rabbits to another soluble streptococcal product occurring in reduced culture filtrates. The results presented here indicate that the active factor in these reduced filtrates is streptolysin O. Preliminary studies on the nature of the material in the lesion extract which is responsible for potentiating the action of streptolysin O are also reported.

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SERUM GLYCOPROTEIN STUDIES IN EXPERIMENTAL BRUCELLOSIS OF THE GUINEA PIG

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The serum protein response in several different infections has been studied by a number of investigators employing electrophoretic or salting-out methods. Gutman[1] has summarized the significant data in his excellent review. In contrast, few data are available on the effects of infectious diseases on the carbohydrate-containing proteins of serum. The response of the serum glycoproteins in clinical and experimental infections has been investigated most thoroughly in tuberculosis.[2–9] Marked increases in the protein-bound carbohydrates of serum have been reported in human tuberculosis[2–7] and in experimental tuberculosis of guinea pigs[8] and of rabbits.[9] Elevations of serum mucoprotein levels have been observed in pneumonia,[10] bacterial endocarditis,[11] poliomyelitis,[12] and osteomyelitis.[12]

Notwithstanding the public health and economic importance of brucellosis, there is a paucity of information with regard to the effects of the disease on the serum proteins of the host. Previous studies on the serum protein response of the host to infection with *Brucella* have been concerned primarily with the effect of the disease on the development of serum agglutinins[13] and bactericidal substances.[14] Inasmuch as the guinea pig is widely employed as an experimental animal in evaluating the efficacy of chemotherapeutic agents in the treatment of brucellosis, a study of the serum glycoproteins and proteins in the experimental disease was undertaken. The present communication reports on the effects of the disease on the serum con-

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centrations of total polysaccharide, mucoprotein polysaccharide, γ-globulin polysaccharide, total protein, and γ-globulin protein in guinea pigs experimentally infected with a virulent strain of *Brucella suis*.

EXPERIMENTAL

One hundred and twenty-nine male guinea pigs with an average weight of 400 g were inoculated intraperitoneally in the inguinal region with approximately 1.3×10^7 cells of *B. suis*, strain 364.* The animals were housed, five per cage, in an air conditioned room and were maintained on a diet of rabbit pellets plus fresh greens, and tap water ad libitum. Two weeks after inoculation the animals were separated into four groups. The groups were injected intramuscularly at 12-hour intervals for 2 weeks with 1.25 mg ACTH,† 1.25 mg cortisone,‡ 5.0 mg terramycin,§ and 0.25 ml 0.85% sodium chloride, respectively. Six weeks after treatment the animals were sacrificed by exsanguination from the heart and air embolism. The serums were separated and stored at −20 C until the chemical analyses could be conducted. Blood was also obtained by cardiac puncture from 44 normal animals and treated in a similar manner.

Chemical analyses.—Gamma globulin was isolated from serum by an adaptation of a low temperature-ethanol procedure.[15] To 1 ml of serum were added 3 ml of distilled water, and the pH of the solution was adjusted to 7.5 to 7.7 with 0.05 *M* acetic acid or 0.05 *M* disodium phosphate. The solution was cooled to −1 C in a cracked ice-calcium chloride bath. Fifty per cent ethanol (v/v) precooled to −5 C was added, dropwise, with automatic, continuous stirring until the ethanol concentration attained 20% (v/v). Stirring was continued for 5 minutes following the addition of the ethanol. The mixture was allowed to stand at −1 C for 30 minutes. The protein precipitate was recovered by centrifugation in an International refrigerated centrifuge, model PR-1, for 30 minutes at 3500 r.p.m. The supernatant fluid was decanted and the γ-globulin dissolved and made-up to 10 ml with 0.85% sodium chloride. Gamma globulin polysaccharide was determined in the following manner. A 2 ml aliquot of the γ-globulin solution was added, dropwise, to 10 ml of absolute ethanol at room temperature. The mixture was allowed to stand 10 minutes and the precipitate recovered by centrifugation at 1800 r.p.m. for 10 minutes. The ethanol was decanted and the precipitate was drained by inversion of the tube. One ml of distilled water was added and the hexose content of the precipitate determined by an orcinol reaction.[8] The protein content of the γ-globulin fraction was determined by the biuret method of Weichselbaum.[16] To a 2 ml aliquot of the γ-globulin solution were added 3 ml of 0.85% sodium chloride followed by 5 ml of biuret reagent.[16] The contents of the tube were thoroughly mixed and placed in a water bath at 32 C for 30 minutes. Standards containing 2 mg and 4 mg of crystalline, bovine, serum albumin and a reagent blank were treated in a similar manner. The protein content of the γ-globulin fraction was then determined from Klett-Summerson photoelectric colorimeter readings, employing the no. 54 (green) filter.

For the determination of the serum concentrations of total polysaccharide, mucoprotein polysaccharide, and total protein, 2 ml of serum were diluted to 20 ml with 0.85% sodium chloride. Total polysaccharide was determined in a 1 ml aliquot of the diluted serum by the procedure of Weimer and Moshin.[8] Five ml of the diluted serum were employed in the estimation of mucoprotein polysaccharide by a modification of the method of Winzler.[8] The total serum polysaccharide and mucoprotein polysaccharide values were employed as indices of the levels of total serum glycoprotein and serum mucoprotein, respectively. Total protein was determined in a 1 ml aliquot of the diluted serum by the biuret method of Weichselbaum.[16] All chemical analyses were carried out in duplicate.

Analysis of results.—The mean, standard error of the mean, *t*, and probability values were determined by the methods outlined by Snedecor.[17]

RESULTS AND DISCUSSION

A summary of the treatment regimens, average weights, and the gross

* Obtained through the courtesy of Dr. W. W. Spink, Department of Medicine, University of Minnesota Medical School.

† Obtained through the courtesy of Mr. S. S. Kingman and Mr. R. C. Bruner, United Laboratories Ltd., Pasadena, California.

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TABLE 1.—*Summary of gross pathologic, immunologic, and bacteriologic observations.*

	B	C	D	E
Group	Infected	Infected	Infected	Infected
Number of animals	20	28	21	20
Treatment	0.5 ml saline/day	2.5 mg ACTH/day	2.5 mg cortisone/day	10.0 mg terramycin/day
Average weight before infection (g)	398	420	408	387
Average weight at autopsy (g)	456	453	462	480
Average*,† index of brucellosis	16.3±0.62	15.5±0.69	14.2±0.86	14.1±0.56
P		0.4	>0.05	<0.02
Median agglutinin titer	1:2560	1:2560	1:2560	1:2560
Positive blood cultures (%)	62	50	63	38
Positive spleen cultures (%)	95	100	91	95

* Including the standard error of the mean.
† The liver, spleen, joints, lymph nodes, lungs, epididymides, testes were each assigned a value of 0 to 4 according to the extent of macroscopic brucellosis and the spleen weight was rated 0 to 2. Maximum extent of disease in each animal was 30. Index of brucellosis is the average total for each group.[18]

pathologic, immunologic, and bacteriologic observations is presented in table 1. Results of chemical analyses are shown in table 2. A summary of the relationships between the serum polysaccharides and the serum proteins is presented in table 3.

The data presented in table 1 are reported in greater detail elsewhere[18] and are included in the present communication for correlation with the results of the serum glycoprotein and protein determinations.

Statistically significant increases in the serum concentrations of total polysaccharide, mucoprotein polysaccharide, γ-globulin polysaccharide, total protein, and γ-globulin protein were observed in infected groups in comparison with the mean values of the normal control group.

Approximately 90% of the elevation of the total serum polysaccharide levels in the infected groups can be accounted for by the increased concentrations of mucoprotein polysaccharide plus γ-globulin polysaccharide.

The greatest relative increases occurred in the γ-globulin fractions of the infected animals. The inoculated groups exhibited a twofold increase in γ-globulin in comparison with the normal control group. The elevation in γ-globulin protein in the infected groups was greater than the increase in total serum protein, indicating a decrease in some major component of the serum proteins. Electrophoretic analyses of serums from normal and brucella-infected guinea pigs have demonstrated a decrease in the serum albumin of animals with brucella infection.[19]

18. Boak, R. A., Carpenter, C. M., Drusch, H. E., Miller, J. N., Redlich-Moshin, J. and Weimer, H. E. To be published.

19. Jameson, E. Personal communication.

TABLE 2.—*Summary of results of chemical analyses.*

	A	B	C	D	E
Group	Normal	Infected	Infected	Infected	Infected
Number of animals	44	20	28	21	20
Treatment	None	0.5 ml saline/day	2.5 mg ACTH/day	2.5 mg cortisone/day	10.0 mg terramycin/day
Total serum* polysaccharide (mg %)	110±1.8	154±3.9	148±2.1	149±4.2	154±4.2
P		0.001	0.001	0.001	0.001
Mucoprotein* polysaccharide (mg %)	28±0.8	44±2.4	42±1.3	44±1.7	47±2.9
P		0.001	0.001	0.001	0.001
γ-globulin* polysaccharide (mg %)	22±1.1†	48±1.8	43±1.4	41±1.7	42±1.5
P		0.001	0.001	0.001	0.001
Total serum* protein (g %)	5.3±0.06	6.2±0.15	6.0±0.09	6.0±0.14	6.0±0.08
P		0.001	0.001	0.001	0.001
γ-globulin* protein (g %)	1.3±0.05†	2.6±0.10	2.5±0.10	2.4±0.11	2.4±0.07
P		0.001	0.001	0.001	0.001

* Including the standard error of the mean.
† 21 animals.

TABLE 3.—*The relationships of the serum polysaccharides and the serum proteins.*

	A	B	C	D	E
Group	Normal	Infected	Infected	Infected	Infected
Number of animals	44	20	28	21	20
Treatment	None	0.5 ml saline/day	2.5 mg ACTH/day	2.5 mg cortisone/day	10.0 mg terramycin/day
Total serum polysaccharide÷total serum protein X100 (%)*	2.1±0.03	2.5±0.07	2.5±0.04	2.5±0.04	2.6±0.06
P		0.001	0.001	0.001	0.001
γ-globulin polysaccharide÷γ-globulin protein X100 (%)*	1.7±0.04	1.8±0.03	1.7±0.03	1.7±0.05	1.8±0.03
P		0.05	1.00	1.00	0.05
γ-globulin protein÷total serum protein X100 (%)*	24.5±1.02	41.9±1.15	41.6±1.11	40.0±1.32	40.0±0.91
P		0.001	0.001	0.001	0.001
Mucoprotein polysaccharide÷total serum polysaccharide X100 (%)*	25.5±0.53	28.6±1.10	28.4±0.94	29.6±0.99	30.5±1.18
P		0.01	0.01	0.001	0.001
γ-globulin polysaccharide÷total serum polysaccharide X100 (%)*	20.0±0.53	31.2±1.22	29.1±0.97	27.5±0.98	27.3±0.97
P		0.001	0.001	0.001	0.001

* Including the standard error of the mean.

The increases in serum glycoproteins and proteins paralleled to a certain extent the amount of macroscopic brucellosis. Only group E, which received terramycin, exhibited a significant difference in the average index of brucellosis when compared with the infected, saline-treated animals of group B. All inoculated groups had well-established, extensive disease with the typical pathology of *Br. suis* infection.[20] Apparently the serum protein response in the pathologic conditions described above is of such magnitude that small differences in the amount of disease do not alter appreciably the degree of the response.

The similarity in the serum protein response of the inoculated groups to the experimental infection was also reflected in the median values of the serum agglutinin titers.

The small range in the mean values of the serum constituents among the infected groups suggested that prior treatment had little or no effect on the observed values. Weimer and Redlich-Moshin,[21] however, have demonstrated that the intramuscular administration of ACTH, cortisone, or saline to normal guinea pigs may influence total serum glycoprotein, serum mucoprotein, or total serum protein levels. Apparently the natural progression of the disease in the host in an extended posttreatment period tends to obscure the effects of prior treatment.

A study of the relationships of the serum polysaccharides and the serum proteins provided additional information on the response of the carbohydrate-containing proteins of serum to infection (table 3).

Polysaccharide was computed to comprise 2.1% of the total serum protein of the normal control group (A). Higher percentages of statistical significance were calculated for all groups with brucellosis. Results of the computations indicated that the elevation of total serum polysaccharide reflected not merely the general increase in total serum protein, but also a marked increase in carbohydrate-containing proteins.

A concomitant increase occurred in both the polysaccharide and protein moieties of the γ-globulin fraction of the infected groups, resulting in a fairly constant ratio between the estimated components. Although the heterogeneity of the γ-globulin fraction is well known, it is apparent from the observed ratios in the normal and infected groups

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21. Weimer, H. E. and Redlich-Moshin, J. 1953, Proc. Soc. Exper. Biol. & Med. **84**: 34–37.

that the increases in the polysaccharide and protein components were quite uniform.

The increased percentage of γ-globulin in the total serum protein observed in the guinea pigs with brucellosis has been noted previously in diseases caused by infectious agents.[1] Elevated γ-globulin levels have been associated with hyperproteinemia and are related to the immune response.[1] The percentage of γ-globulin in the serum of the normal animals of group A is in good agreement with the result reported by Svensson,[22] based on electrophoretic analyses, but greater than the value reported by Moore,[23] also obtained by electrophoresis.

Serum mucoprotein polysaccharide exhibited a statistically significant increase with respect to the total serum polysaccharide in the infected groups. A similar observation has been reported for guinea pigs with experimental tuberculosis.[8]

Although a number of workers have studied the serum γ-globulin response in infectious diseases,[1] little attention has been directed to the polysaccharide moiety of the γ-globulin fraction. It was apparent that the protein-bound carbohydrate of the γ-globulin fraction comprised an appreciable amount of the total serum polysaccharide in the normal state and that the percentage was markedly increased in an acute brucella infection.

The studies reported above indicate that the serum glycoprotein and protein responses in experimental brucellosis of the guinea pig are characterized by: (1) an increased concentration of total serum glycoprotein, (2) elevated levels of serum mucoprotein, (3) hyperproteinemia, and (4) hypergammaglobulinemia.

SUMMARY

Total polysaccharide, mucoprotein polysaccharide, γ-globulin polysaccharide, total protein, and γ-globulin protein have been determined in the serum of guinea pigs infected with a virulent strain of *Brucella suis*, and treated with terramycin, ACTH, cortisone, and 0.85% sodium chloride. Statistically significant increases were observed in each of the serum constituents of the infected groups, irrespective of treatment. The relationships of the serum polysaccharides and the serum proteins were investigated.

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INTRACEREBRAL INOCULATION OF MICE WITH NEWCASTLE DISEASE VIRUS

II. THE RESPONSE OF MICE OF DIFFERENT AGES

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The results of intracerebral inoculation of 25 strains of Newcastle disease virus (NDV) into weanling mice were reported in a previous communication.[1] Some of the strains produced rapidly fatal involvement of the nervous system, while others had little effect upon the mice. The growth of strictly egg-adapted or chicken-adapted strains of NDV in weanling mice has not been detected,[2,3] with a possible exception.[4] Kilham[5] was successful in serially passaging the 11914 and Vict strains of NDV in 3-day-old suckling mice.

The finding of such differences in the response to NDV of young and mature mice prompted the present study in which the effect of strains of NDV of various degrees of neuropathogenicity were observed in 4 age groups of mice. In the initial survey no attempts at serial passage were made. The results of serial passage of 4 of these strains in suckling mice are being reported separately.[6]

MATERIALS AND METHODS

Swiss albino mice used in these studies were from (1) the stock colony of the Veterinary Science Department, which had originated from mice obtained from the Rockefeller Institute, and from (2) Carworth Farm stock, also a Rockefeller line of Swiss albino mice (CFW). Three-day-old, 9-day-old, weanling and adult mice were employed. Suckling mice between 3 and 5 days old had little or no hair and an average weight of about 2 g. Nine- to 11-day-old mice had good hair coats and weighed between 5 and 6 g. Effects of litter variation in resistance to injury were reduced by pooling all sucklings of a given age to be inoculated at one time. Animals designated as "weanlings" were between 21 and 45 days old. The adult mice were more than 2 months and usually about 6 months old at the time of inoculation.

Used in the survey were 16 strains of NDV whose histories were given in a previous paper.[1] Nine of the strains were tested on adults; these comprised Herts-E[5], 11914-Reagan, 125-Iowa, Milano, 11914-FD, Miller, Cle, KD-NJ, and GB. These and 7 additional strains were injected into 3-to-5 and 9-day-old suckling mice. They were: Ixta, Roakin, 23-Iowa, Len, B1, Naj, and Lab.

Infected, freshly harvested allantoic fluids from individual eggs treated as previously described[1] served as inoculums. Replicate tests in suckling mice with fluids stored for about 1 week at 8 C or for longer periods at −20 C gave the same results as did the freshly harvested fluids. Strains injected into suckling and adult mice usually had been through the same number of egg passages as those which were injected into weanlings. Data on inoculation of weanlings were transposed from the previous paper with further replications of tests in some cases. Normal allantoic fluids were inoculated as controls into mice of every age.

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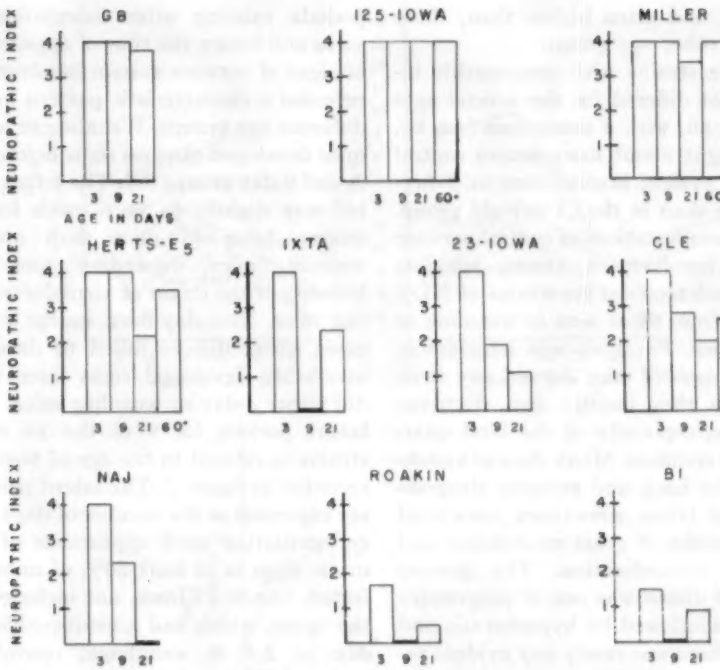

FIG. 1.—Effect of age upon susceptibility of mice to NDV.

Three-day-old mice received 0.02 ml of inoculum in the right cerebral hemisphere with a 26 or 27 gauge needle and a 0.25 ml tuberculin syringe. Nine day mice were similarly inoculated with 0.03 ml of material. Weanling or older mice were anesthetized with ether prior to inoculation and given 0.05 ml of inoculum. All animals were observed for 11 to 15 days after inoculation.

Neuropathic indices, determined on the basis of signs as previously described,[1] were calculated for all groups of mice. The highest value, 4, denotes that all animals in a group died. A zero value indicates that there were no detectable reactions.

RESULTS

The neuropathic indices for the four ages of mice are given in figure 1. The figure represents averages of replicate tests. With all except 2 of the 16 strains which were inoculated into 3-day-old mice at least one repeat test was made; with 4 strains there were 3 or 4 trials. In 9-day-old mice 9 strains were tested twice and 7 were tested only once. Replications were made on all strains injected into weanling and adult mice. The number of mice in the 3- and 9-day groups depended upon the size of the litters available on the day of inoculation. The number injected in any test group usually lay between 5 and 9, although as few as 4 and as many as 11 mice were used. There were between 7 and 9 mice in the weanling and adult test groups. Of the strains not included in the figure, Milano, 11914-FD and 11914-Reagan were as highly lethal as was 125-Iowa. Among the strains of intermediate and low neuropathogenicity the Lab strain closely resembled Ixta in its action and strains KD and Len resembled 23-Iowa. Normal allantoic fluids inoculated intracerebrally did not produce adverse effects in mice of any age.

The neuropathic indices obtained for 3- and 5-day-old mice were always as

high as, and often higher than, those for any other age group.

Of the strains with neuropathic indices that differed for the several ages of mice, all, with 4 exceptions (see fig. 1), brought about more severe central nervous system involvement in 9-day-old mice than in the 21-day-old group.

The manifestations of central nervous system involvement among suckling mice which received the strains of NDV differed from those seen in weanling or adult mice. Paralysis was uncommon. If the 3-day-old mice showed any obvious signs they usually died. Extreme weakness, especially of the hind quarters, was common. Many showed hunching of the back and extreme sluggishness, the latter sometimes associated with episodes of great excitability and marked incoordination. The general course of illness was one of progressive weakness followed by hypothermia and death. There was rarely any evident reaction among the 3-day-mice within the first 24 hours such as occurred among the 21-day mice. Nine-day-old mice exhibited the same general type of signs as did 3-day-old mice, although paralysis and hyperirritability were more prevalent than in the younger group.

Weanling mice which received the lethal strains died quickly, often before the 20th hour. Those surviving for longer periods showed signs of severe central nervous system involvement, including spastic paralysis, tremor, and clonic spasms. Weanling mice which received the milder strains failed to show obvious signs or reacted with immediate, mild and transitory nervous reactions. However, a small percentage of this group became paralyzed and died between the 3rd and 8th day. Mice 2 to 6 months of age were found to be somewhat more susceptible to the neuropathic action of NDV than weanling mice, although the clinical manifestations were of the same general nature in both.

Comparison of the length of the latent periods existing after inoculation of virus and before the time of appearance of signs of nervous system involvement revealed a characteristic pattern in the different age groups. Weanling or adult mice developed obvious signs before the 3- and 9-day groups did. The latent period was slightly to very much longer among 3-day-old mice than among weanling mice, depending upon the lethality of the strain of virus for weanling mice. Nine-day mice, except in the cases where disease failed to develop, invariably developed signs later than did either 3-day or weanling mice. The latent periods for 9 of the 16 virus strains as related to the age of host are recorded in figure 2. The latent periods are expressed as the number of days after inoculation until appearance of climatic signs in at least 50% of mice affected. Strain 23-Iowa, not included in the figure, which had a neuropathic index of 2.3 in weanlings, resembled strains Miller and 11914 in the length of latency. Strains Naj, Kd, Bl, and Roakin, also excluded from the figure, either had no effect on weanling mice or signs were so slight and infrequent as to prevent accurate determination of a characteristic latent period.

DISCUSSION

In these studies replications of different strains were limited in number and the size of these test groups was small. The purpose was to determine a general pattern in the response of mice of different ages to NDV, rather than a statistical evaluation of the differences between strains. The pattern of age reactivity has been shown. All 16 strains inoculated into 3-day mice manifested a greater pathogenic activity combined with a lengthened latent period until appearance of signs, as compared to the effects induced in weanling and adult mice. An equally consistant result was obtained with the 16 strains in 9-day-old mice, which were invariably more re-

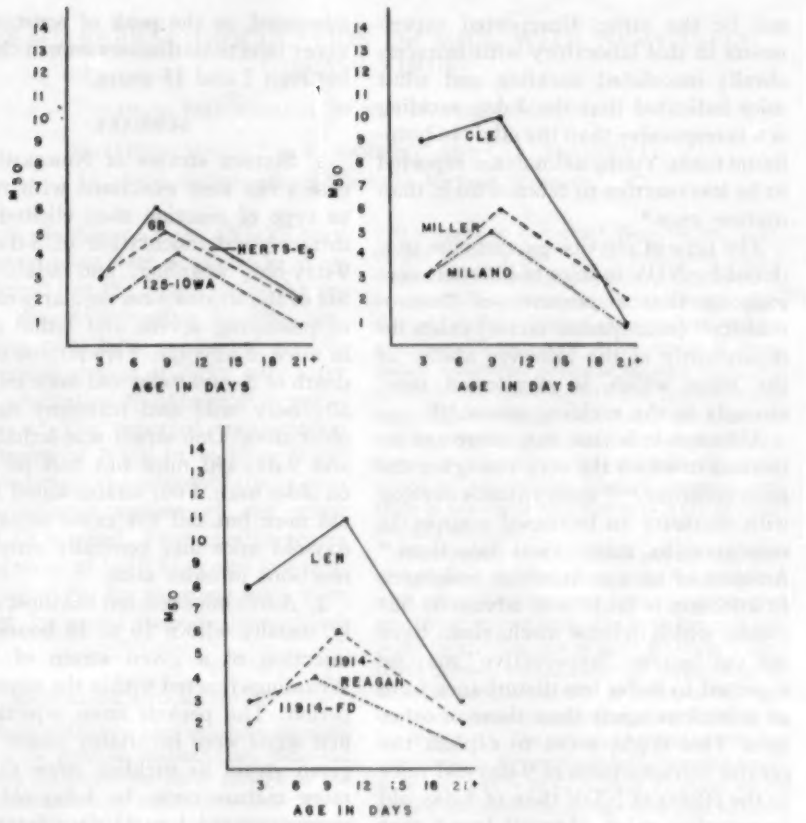

FIG. 2.—Correlation of age of mouse with latent periods shown by NDV strains.

sistant than 3-day-old mice to infection and more resistant than weanlings to the toxic effects of NDV. Strains of NDV consequently exhibited a latent period consistently longer in this age group than that in 3-day-old mice or weanlings. Weanlings and adults reacted the most rapidly to the toxic effects of NDV and were more resistant to infection than any group.

Burnet[7] has suggested that the host defense mechanisms may be perfected to such a degree in the young adult human being that there is an overactive response to infective and other stimuli which may be so severe as to cause considerable injury to the host. This may be a general phenomenon occurring among all mammals.

The skin and lung especially have been shown to be relatively unreactive in the young and to develop with maturity an increased capacity to respond to injury.[7,8] The central nervous system would appear to be another system which follows this pattern, although the basis for increased reactivity may

7. Burnet, F. M. 1940, Biological Aspects of Infectious Diseases, Cambridge, Cambridge University Press.

8. Baumgartner, L. 1934, Yale J. Biol. 6: 403–434.

not be the same. Unreported experiments in this laboratory with intracerebrally inoculated suckling and adult mice indicated that the 3-day suckling is less responsive than the adult to botulinum toxin. Young animals are reported to be less reactive to tetanus toxin than mature ones.[9]

The type of physiologic response produced by NDV in mice of different ages suggests that a property of "neurotoxicity" (neuropathic factor) exists independently of the infective ability of the virus which is manifested most strongly in the suckling mouse.[2-5]

Although it is true that there are infections to which the very young are the most resistant,[10,11] most animals develop with maturity an increased nonspecific resistance to many viral infections.[12] Animals of an age at which resistance to infection is fairly well advanced but among which defense mechanisms have not yet become "hyperactive" may be expected to suffer less disturbance from an infectious agent than those of other ages. This might serve to explain the greater refractoriness of 9-day-old mice to the effects of NDV than of 3-day-old or weanling mice. Howes[13] found that mice 8 to 11-days-old were more resistant than mice of any other age to two strains of Coxsackie virus capable of infecting adults. There is an apparent parallel in human medicine, and for which the same explanation has been advanced, in the peak of resistance to many infectious diseases seen in children between 2 and 15 years.[7]

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11. Whitney, E. 1951, Proc. Soc. Exper. Biol. & Med. **78**: 247–250.
12. Sigel, M. M. 1952, Ann. Rev. Microbiol. **6**: 247–280.
13. Howes, D. W. 1953, Austral. J. Exper. Biol. **30**: 423–436.

SUMMARY

1. Sixteen strains of Newcastle disease virus were examined with respect to type of reaction they elicited after intracerebral inoculation of 3-day-old, 9-day-old, weanling, and adult mice. Six of the strains were regularly capable of producing severe and lethal effects in mice of any age. Five strains caused death of 3- and 9-day-old mice but usually only mild and transient signs in older mice. One strain was lethal in 3- and 9-day-old mice but had no effect on older mice. Four strains killed 3-day-old mice but did not cause signs in 9-day-old mice and generally only mild reactions in older mice.

2. Adult mice reacted the most rapidly, usually within 16 to 48 hours after injection of a given strain of virus. Weanlings reacted within the same time period. The periods from injection to first signs were invariably longer for a given strain in suckling mice than in more mature ones. In 3-day-old mice signs appeared 1 to 11 days later than in weanlings and adults. In 9-day-old mice the appearance of signs was delayed 2 to 12 days.

3. In weanlings and adults spastic paralysis commonly followed injection of a lethal strain, although the mice were more often found dead. Less toxic strains frequently caused only transient incoordination, ruffling of hair, and anorexia.

Suckling mice always succumbed after developing symptoms. Paralysis was uncommon, hyperirritability, sluggishness, and progressive weakness being the most characteristic feature of infection.

INTRACEREBRAL INOCULATION OF MICE WITH NEWCASTLE DISEASE VIRUS

III. SERIAL PASSAGE OF NDV IN SUCKLING MICE

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Newcastle disease virus (NDV) may elicit nervous system response in mice of all ages,[1] but the character of the response differs according to the age. Weanling mice have supported growth of American strains of NDV only after initial adaptation to hamsters[2] or suckling mice.[3] The reported transmission of 2 strains of NDV in suckling mice[4] suggested that the adaptability of several additional strains of the virus should be studied. Four strains representing virus of both high and low neuropathogenicity for weanling mice were selected for serial passage in suckling mice.

MATERIALS AND METHODS

The Swiss albino mice used were from the same source as for previous studies.[1] Only 3-day-old mice were inoculated for serial passage of the virus unless specifically stated otherwise.

The histories of the 4 strains of NDV are recorded in a previous report.[5] Of the 3 strains lethal for weanling mice, 2, 125-Iowa and 11914-Reagan, had undergone previous mammalian passage. Strain 11914-Reagan was reported to be well adapted to hamsters.[2] Nevertheless, 2 attempts to carry this line through more than 6 consecutive hamster passages were not successful. Strain 125-Iowa had been carried through alternate pig embryo-chicken embryo-sheep embryo passages without record of the degree of mammalian adaptation produced by such manipulations.[6] Strain 11914-BAI-15 had not undergone mammalian passage. Strain Naj, which is innocuous for weanling mice, had been isolated from a human eye infection and subsequently maintained by egg passage. Limited attempts were made to passage several strains of NDV in weanling and adult mice.

The material used for serial passage consisted of brains of suckling mice previously injected with virus. Serial passage was also attempted employing mature mice. The inoculum was prepared by grinding one or more brains in a glass tissue grinder, adding sufficient tryptone broth to make an approximate 10^{-1} suspension‡ and centrifuging the suspension at about 2000 r.p.m. for 20 minutes. The supernatant fluid constituted the inoculum. Between passages the entire brains were stored at −20 C. A 26 or 27 gauge needle and a ½ ml tuberculin syringe were used to inject 0.01 to 0.02 ml of the inoculum intracerebrally into suckling mice and 0.03 ml into adult mice.

Eight successive passages of brains of normal 3-day suckling and normal weanling mice failed to activate any latent virus which might have been present in the mice.

RESULTS

Intracerebral passage in suckling mice of 3 neuropathic strains of virus

Strain 11914-BAI-15 was carried through 16 serial passages in the brains

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6. Hofstad, M. S. 1949, Personal communication.

‡ Brains of suckling mice were found to average 0.10 to 0.13 g for 3-day mice and 0.15 g for 5-day mice. For each day up to 10 days of age approximately 0.01 g should be added to the brain weight. Ten day mouse brains averaged 0.2 g. Brains of 4- to 6-week-old mice averaged 0.4 g.

of suckling mice. The infected egg fluids used for inoculating the first series produced severe nervous signs or death within 4 days. By the 3rd passage the period until appearance of such signs was reduced to 2 or 3 days and, in general, remained the same through the 16th passage. The mice used for the 6th to 8th passages were 4 to 6 days of age. The incubation period in these mice was lengthened somewhat. Material from several passages between the 1st and the 16th killed chicken embryos. The embryo LD_{50} (ELD_{50}) increased to a maximum of 10^6 ELD_{50}'s per 0.1 g of mouse brain in the 6th passage and decreased to 10^1 or 10^2 ELD_{50}'s after the 11th passage. Allantoic fluids from dead embryos invariably produced hemagglutination.

Some mice of all passages exhibited convulsive seizures shortly before death. The development of convulsions was preceded by a period during which the animals were usually very sluggish and extremely "hunched-up," although hyperirritability was noticed sometimes. One of the first signs was loss of coordination. The incoordination and weakness progressed rapidly to complete ataxia. Cases of actual paralysis, however, were not observed before the 4th or 5th passages. Paralysis occurred occasionally after the 5th passage.

Strain 11914-Reagan was carried through 18 successive suckling mouse brain transfers. The signs were generally the same as those associated with strain 11914-BAI-15 infection. The incubation period was 2 or 3 days in 3-day-old mice. As with strain 11914 the incubation period was lengthened when 6-day mice were employed. The highest ELD_{50} titer was greater than $10^{3.5}$ in the 9th passage.

Strain 125-Iowa, although lethal for both weanling and suckling mice when administered as undiluted infected allantoic fluids, was the only one of the 4 strains which was difficult to passage serially in 3-day-old mice. Virus in the infected fluids caused severe signs or death within 3 days. The incubation period was lengthened during the first 7 passages to 6 to 10 days. Usually some mice in the group remained unaffected. No mice died in the 8th passage. However, 7th passage mouse brain suspension killed chicken embryos with the production of hemagglutinins in the allantoic fluids. The allantoic fluid from one egg was used for further passage. Ten successive passages were made in this series before the line was lost again. The incubation period had decreased to 3 to 7 days by the 6th passage but increased thereafter. Strain 125-Iowa seldom developed titers greater than 10^2 ELD_{50}'s per 0.1 g of mouse brain in either series.

Mice affected with 125-Iowa manifested the most striking nervous signs of any. Hyperexcitability, muscular spasms and convulsions were common signs. Stimulation of the animal sometimes would cause severe convulsions. This strain of virus produced a higher proportion of marked paralytic signs than did the others.

Intracerebral passage in suckling mice of a non-neuropathic strain of virus

Strain Naj which had failed consistently to produce signs in weanling mice was passaged through 16 serial transfers in 3-day suckling mice. Infected allantoic fluid killed the suckling mice in 4 to 10 days. In the second, third and fourth passages mice became ill after an incubation period of 3 to 5 days. From the 5th passage onward the incubation period was usually 3 days and the mortality 100%. All passage material which was injected into embryonating eggs caused death of the embryo and de-

velopment of hemagglutinins in the extraembryonic fluids. The highest titers obtained were 10^3 and 10^4 ELD_{50}'s between the 3rd and 7th passages. After the 7th passage there was a gradual decline in titer.

Suckling mice which had received strain Naj developed incoordination but convulsions or paralysis were rarely seen. Starting about a day before death the mice became progressively more sluggish and hunched. Finally, they developed ataxia and lay on their sides although there was still movement of the limbs. Transfer of the brain from asymptomatic mice, previously inoculated with strain Naj, failed to affect the recipient mice. Passage of the brains of prostrate mice invariably led to the death of the recipient mice. These preliminary tests indicate that the titer of NDV in the brain may be highest at the time that signs of disease appear even though these may not develop for several days.

A limited number of titrations of 3 of the mouse-passaged strains have been made in suckling mice. After 16 passages Naj virus killed all mice receiving 0.01 ml of the 10^{-1} dilution and affected 2 out of 8 receiving the 10^{-3} dilution. Complete neutralization of the 16th passage Naj virus with homologous antiserum was demonstrated by mouse injection.

Titration of 18th passage 11914-Reagan virus yielded results similar to those obtained from titration of Naj in mice. Material from the 11th serial mouse brain passage of 11914-BAI-15 killed sucklings in 10^{-1} but not in 10^{-3} dilutions.

After these experiments were terminated subsequent rapid serial passage of the 11914-Reagan and Naj strains resulted in a temporary increase in ELD_{50} titer, followed by a gradual decline. Strain 11914-Reagan had completely lost its ability to kill chicken embryos by the 45th suckling mouse passage. Rapid serial passage also resulted in some increase in the mouse LD_{50} titer. It is possible that the 125-Iowa line could have been adapted by such a procedure. Wenner[7] reported complete loss of pathogenicity for chicken embryos by NDV strain 11914 after only 3 passages in monkeys. Kilham[4] found that strain 11914 had lost its pathogenicity for chicken embryos after 11 passages in suckling mice.

Preliminary efforts to passage egg-adapted strains RO and 125-Iowa in weanling or adult mice were unsuccessful, as were attempts with suckling mouse adapted 11914-Reagan and Naj.

DISCUSSION

It is well known that many viruses proliferate more readily in very young hosts than in older ones.[8] Among the neurotropic viruses, NDV may be compared with rabies virus[9] and herpes virus[10] in that adult mice are less able than sucklings to support proliferation of cerebrally inoculated virus. Other neurotropic viruses grow equally well in both young and adult hosts if injected by the cerebral but not by peripheral routes.[11,12] The observations made here and in the preceding paper than suckling mice provide a better substrate for growth than mature mice for NDVwhile reacting less rapidly to "toxic" effects of the virus, indicates the complexity of the virus-host interaction.

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The independence of "toxic" and growth processes in some virus diseases is indicated by studies with the psittacosis-lymphogranuloma groups of viruses.[13] The "toxicity" of certain strains of this group for mice is independent of any proliferative processes, since the toxic effect is not eliminated by antibiotic agents which inhibit growth of the virus. Evidence of independence of toxigenic and infective principles of influenza virus has been supplied by Stuart-Harris[14] and Francis and Moore,[15] both of whom passaged the WS strain cerebrally through 11 series of mice before any signs were produced. Similarly, Hirst[16] found that strains of influenza A virus could be passaged in the mouse lung and that they would grow to maximum titer at each passage, although clinical signs of infection were not produced before the 4th or 5th passage.

Other work has suggested that among strains of NDV and influenza possessing "neurotoxicity" the toxigenicity and proliferation of such viruses in the mouse are controlled by partly interdependent mechanisms. Wagner[17] and Ginsberg[18] have shown that adsorption of virus to susceptible cells is necessary before "toxic" manifestations of influenza virus or NDV can be induced in the brain or the lungs. Schlesinger[19] has shown that an uncompleted growth process is initiated on inoculation of non-neurotropic influenza virus into mice and has suggested that it is related to the "toxicity" of the virus. The two-step growth cycles reported for strains of influenza A virus and the Victoria strain of NDV in mice[20] might have a similar relation to "toxicity." There is no evidence, however, that adsorption to cells and initiation of uncompleted growth cycles does not occur with equal facility with the non-neuropathic strains since the Victoria strain has low "toxicity" for adult mice.

The present study has revealed further indications of the actual independence of the characters of infectivity and "neurotoxicity" in NDV. A strain which rapidly killed weanling mice was not always more readily infective for suckling mice than was a nonlethal one. Strain Naj did not affect weanling mice, while 125-Iowa was usually lethal; both appeared to have a poor initial potential for growth in the suckling mouse brain, as evidenced by the long incubation periods in the first passages. However, in subsequent passages Naj adapted itself well, while 125-Iowa did not.

The highly neuropathic strains, 11914-Reagan and 11914-BAI-15, both originated from strain 11914 and appear to possess the same high degree of adaptability to the suckling mouse.

Working with strains of NDV differing in pathogenicity for chickens, Sinha and Hanson[21] found that the MK strain of NDV, which seldom killed chickens, reached a higher titer in the brains of birds exposed by aerosol than did strain CG, which invariably killed chickens.

Schlesinger[22] and Karzon and Bang[23] have shown that the rate of viral multi-

13. Manire, G. P. and Meyer, K. F. 1950, J. Infect. Dis. **86**: 233–240.
14. Stuart-Harris, C. H. 1939, Lancet, **1**: 497–499.
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17. Wagner, R. R. 1952, Brit. J. Exper. Path. **33**: 157–167.
18. Ginsberg, H. S. 1951, J. Exper. Med. **94**: 191–211.
19. Schlesinger, R. W. 1950, Proc. Soc. Exper. Biol. & Med. **74**: 541–548.
20. Cairns, H. J. F. 1951, Brit. J. Exper. Path. **32**: 110–117.
21. Sinha, S. K. and Hanson, R. P. 1952, J. Infect. Dis. **91**: 276–282.
22. Schlesinger, R. W. 1949, J. Exper. Med. **89**: 491–505.

plication is important in determing the virulence of western equine encephalomyelitis virus and NDV. Differences in the degree of pathogenicity for mice of some strains of influenza virus have been demonstrated to be due to differences in length of the lag phase.[24] Differences in invasive power may also influence the virulence of NDV strains.[23]

On the basis of evidence presented in these two reports it is postulated that "neurotoxic" and infective properties of NDV constitute separate functions of the virus. The adsorption of virus onto cells and proliferation or initiation of partial growth cycles may occur regardless of whether a virus strain possesses "neurotoxicity" for the host. With NDV the final outcome of an infectious process may depend on a number of factors such as the rate of growth, the invasiveness and the "neurotoxicity" of the strain.
virus were transferred for 10 to 18 serial passages in the brains of 3-day-old mice.

SUMMARY

1. Four strains of Newcastle disease Three of the 4 strains were lethal for weanling mice prior to passage in suckling mice but apparently unable to multiply in the brains of the older mice. Two of these lethal strains were adapted readily to suckling mice, the third only with difficulty. One strain was unable to produce any untoward effects in weanling mice prior to passage in suckling mice. However, it was easily adaptable to suckling mice.

2. The three easily adapted strains attained titers in mouse brains of 10^4 to 10^6 LD_{50}'s per 0.1 g brain tissue between the 3rd and 9th passages, as determined by chicken embryo inoculation. The less easily adaptable strain seldom attained titers greater than 10^3. As passages in mice were continued the titer for chicken embryos underwent a gradual decrease.

3. The strains did not attain titers for suckling mice greater than 10^3 LD_{50}'s per 0.01 ml of inoculum.

4. The results are discussed with regard to the possibility of the distinct identities of the infective and neurotoxic properties of NDV as demonstrated by the type of response to these two properties of the virus in mice of different ages.

23. Karzon, D. T. and Bang, F. B. 1951, J. Exper. Med. **93**: 267–284.
24. Davenport, F. M. and Francis, T. 1951, J. Exper. Med. **93**: 129–137.

SPECIFIC IDENTIFICATION OF *BACILLUS ANTHRACIS* BY MEANS OF A VARIANT BACTERIOPHAGE

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Cowles,[1] in 1931, described a bacteriophage which was active on *Bacillus anthracis* but not specific for that organism. In 1951 McCloy[2] described an atypical bacillus, strain W, from which she had isolated a bacteriophage. This phage was found to attack all of 171 strains of *B. anthracis* and 2 of 54 strains of *Bacillus cereus* which were tested. Eighteen other species of bacilli tested by her were not lysed. However, the phage failed to produce lysis of smooth variants of the anthrax organisms. She believed that the strain W bacillus was probably an atypical *B. cereus* strain or an unusual *B. anthracis* organism. In a later paper[3] she described this bacteriopage as consisting of a predominant β form and a rare α mutant, each arising from a common precursor. Beta phage was capable of attacking strains of *B. anthracis* but not strain W, while the rare mutant α phage lysed and could be propagated on strain W. The α and β phages were serologically identical.

It is the purpose of this paper to describe a new variant phage (γ), isoloated from the original strain W bacteriophage, which shows greater promise in the identification of *B. anthracis*. This γ phage has several properties which were not exhibited by the original strain W phage, namely: the ability to lyse smooth forms of *B. anthracis*, failure to lyse any of the strains of *B. cereus* which were tested, and the characteristic of being propagated upon and of lysing the original strain W organism.

METHODS AND MATERIALS

By chance it was discovered that a variant phage could be obtained by the procedure given below. The mechanism of the process has not been determined. The γ phage was prepared by incubating a heart infusion broth (Difco, HIB) culture of the lysogenic strain W organism for 30 minutes at 37 C, adding 0.1 ml of strain W bacteriophage, reincubating for 30 minutes at 37 C, and then heating to 70 C for 10 minutes, followed by cooling to 37 C and continuing incubation at this temperature for 24 hours. At the end of that time the broth was filtered through a Seitz filter to separate the phage.

The γ phage was propagated by growing it either on strain Ohio or strain Davis *B. anthracis*. Heart infusion broth tubes (10 ml) were inoculated with a loopful (4 mm) of a 24-hour culture of the host organism. These cultures were incubated at 37 C for 2 hours and 0.1 ml of phage (γ) was added. Incubation at 37 C was continued for an additional 18 hours, at the end of which time the cultures were filtered through a Seitz filter and the phage was stored in the refrigerator for future use. Each filtrate was titrated immediately after filtration and at vari-

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2. McCloy, E. W. 1951, Studies on a lysogenic bacillus strain. I. A bacteriophage specific for *B. anthracis*. J. Hyg. **50**: 114–125.
3. McCloy, E. W. 1951, Unusual behavior of a lysogenic bacillus strain. J. *Gen. Microbiol.* **5**: xiv–xvi.

ous intervals during storage, using the tenfold dilution method. During this investigation a preparation of W phage and a preparation of γ phage were propagated on Davis and Ohio strains. Titers were approximately 1×10^7 and 2×10^8 per ml for the W and γ phages, respectively.

The sensitivities of all of the bacillus strains to γ and W phages were tested by applying a drop of the undiluted filtrate to the test strains inoculated either on heart infusion agar (HIA) containing 10% mule serum or on nutrient agar plates (Difco). Usually cultures were spread on freshly prepared nutrient agar plates. When these were dry, phage was inoculated on 6 to 8 replicate areas on each plate by means of a 4 mm wire loop, and the plates were incubated for 18 hours and examined for lysis. Controls were prepared in a similar manner, using the parent W phage and sterile broth.

The smooth variants of *B. anthracis* strains were prepared by a variation of the method of Chu.[4] Plates of HIA containing serum were inoculated with appropriate cultures and incubated in an atmosphere of 60 to 80% CO_2. The smooth cultures which were obtained were transferred for 14 generations under these conditions, and the S_{14} generation was used in the phage experiments. A loopful of a 24-hour culture of each of these smooth forms was placed in HIB and incubated at 37 C for 2 hours. One-tenth ml of each of the latter cultures was spread on a HIA plate, which, after drying, was inoculated with phage as described previously. Before these plates were examined they were incubated at 37 C for 18 hours under a high CO_2 tension.

A total of 264 strains of the genus *Bacillus* were used. These consisted of the following: 43 strains of *B. anthracis* (including 2 strains later shown to be *B. cereus*), 34 of which had been isolated from outbreaks of anthrax in this country and sent to the Communicable Disease Center Laboratories from various state health departments, 7 were stock strains, and 2 were obtained from the American Type Culture Collection; 87 strains of *B. cereus*, 83 of which were isolated from the air during 1952 and 1953 in Chamblee, Georgia, and 4 obtained from other sources; 44 strains of *Bacillus megatherium*, of which 41 were isolated at the Communicable Disease Center Laboratories, and 3 were obtained from other laboratories. The remainder of the species consisted of 10 strains of *Bacillus pumilis*, 26 of *Bacillus subtilis*, 28 of *Bacillus mycoides*, 10 of *Bacillus coagulans*, 11 of *Bacillus firmus*, 3 of *Bacillus licheniformis* and 2 strains of *Bacillus circulans*, all of which were obtained from the stock collection in the Communicable Disease Center Laboratories. All cultures were tested biochemically and morphologically according to the procedures described by Smith, Gordon and Clark.[5] The egg-yolk test of McGaughey and Chu[6] was employed as an additional criterion for classification. Pathogenicity was determined in mice, and motility was studied by using Gard's[7] "swarming phenomena" and "transmigration tests."

Six of the 43 cultures of *B. anthracis* which were screened deserve special mention because of their ability to form smooth variants under high CO_2 tension on serum agar. The history of these six strains, which were employed extensively throughout these studies, is given below:

Ohio—obtained from the Ohio Department of Health; isolated in 1952 from an outbreak of anthrax due to bone meal imports from Belgium.

North Carolina—isolated in 1953 from an outbreak of anthrax in Monroe, North Carolina and found to be lysogenic.[8]

1007 and 1014—received from Dr. Ruth Gordon and found by her to be pathogenic.

V-762 and NV-767—received in 1952 from the Florida State Board of Health, where they were isolated from outbreaks of anthrax on two different ranches in Florida.

RESULTS

With two exceptions, all rough strains of *B. anthracis* which were tested were lysed by both the γ phage and phage W. These two were *B. anthracis* Ax 108, isolated from "exudate from swelling on the neck of a cow following vaccine," and

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6. McGaughey, C. A. and Chu, H. P. 1948, The egg-yolk reaction of aerobic sporing bacilli. J. Gen. Microbiol. **2**: 334–340.

7. Gard, S. Ztschr. f. Hyg. u. Infectionskr., 1938, 120, s. 615. Cited by Nordberg, B., Studies of *Bacillus anthracis* in regard to its properties of diagnostic and pathogenic importance. Royal Vet. Col. Sweden, Stockholm, 1951, pp. 37–38.

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TABLE 1.—*Action of W and γ phages on species of the genus* Bacillus.

Species	Number of strains tested	Lysed by W phage	Lysed by γ phage
B. anthracis	41*	41	41
B. cereus	89†	2	0
B. megatherium	44	0	0
B. pumilis	10	0	0
B. firmus	11	0	0
B. subtilis	26	0	0
B. mycoides	28	0	0
B. coagulans	10	0	0
B. licheniformis	3	0	0
B. circulans	2	0	0

* Naturally occurring rough forms.
† Including two strains received as *B. anthracis*; one of these, Ax 108, was received from the Florida State Board of Health, where it had been isolated from a cow following vaccination. Initially the W phage produced "thinning" of growth on this strain. The second strain Ax 141 was resistant to the action of both phages. Both cultures were shown to be mislabeled and should be called *B. cereus*.

B. anthracis Ax 141, isolated from an infected colt on a different farm. These strains were received from the Florida Board of Health in 1953. These two strains of "*B. anthracis*" subsequently were put through extensive morphological, biochemical and pathogenicity tests and found to be *B. cereus*. They gave marked opalescence on egg-yolk plates, were motile by the "swarming" and "transmigration" techniques of Gard[7] and by hanging drop, and proved avirulent to white mice. Biochemically they fitted the criteria of Smith et al[8] for *B. cereus*, including a wide zone of hemolysis on blood agar plates. Table 1 shows the results obtained when a variety of strains of several species of the genus *Bacillus* were tested with the γ and W phages. In all cases undiluted phage was used according to the methods previously described.

The difference in the specificity of the W and γ phages became apparent when their action was tested on six strains of *B. anthracis* grown on serum agar in an atmosphere of CO_2. Most colonies of these anthrax strains were found to be smooth, round, and raised, and consisted of highly encapsulated organisms. Under obliquely transmitted light they appeared translucent. These characteristics were stable through 14 generations of subculturing in CO_2 on heart infusion serum agar plates. Table 2 shows the results of comparative studies of the action of γ and W bacteriophage on smooth and rough colony types of these six strains.

From each of five of the above six strains, seven variant types were isolated, using Chu's[4] technique, and these were subjected to phage action. These variants were assigned the symbols used by Nungester[9] in his description of *B. anthracis* variants, namely: rough (R), rough-smooth (RS), smooth (S), rough-mucoid (Rm), smooth-mucoid (SM), rough-phantom (Rp), and smooth-phantom (Sp). The γ phage was able to lyse all of these forms regardless of their origin, while the W phage lysed only the R forms. Sm and Sp forms were not obtained from strain 1007. The methods

9. Nungester, W. J. 1929, Dissociation of *B. anthracis*. J. Infect. Dis. **44**: 73–125.

TABLE 2.—*Effect of W and γ phages on rough cultures of* B. anthracis *and on smooth-mucoid variants derived from these.*

Strain	Rough		Smooth	
	Phage W	γ phage	Phage W	γ phage
B. anthracis Ohio	Completely clear	Completely clear	Appearance unaltered	Clear area
B. anthracis N.C.	Thinning area	Clear with few discrete colonies	Appearance unaltered	Clear area
B. anthracis 1014	Clear with few discrete colonies	Completely clear	Appearance unaltered	Clear area
B. anthracis 1007	Clear with few discrete colonies	Clear with few discrete colonies	Appearance unaltered	Clear area
B. anthracis NV-767	Thinning area	Clear with discrete colonies	Appearance unaltered	Clear zone with few discrete colonies
B. anthracis V-762	Thinning area	Thinning area	Appearance unaltered	Clear zone with discrete colonies

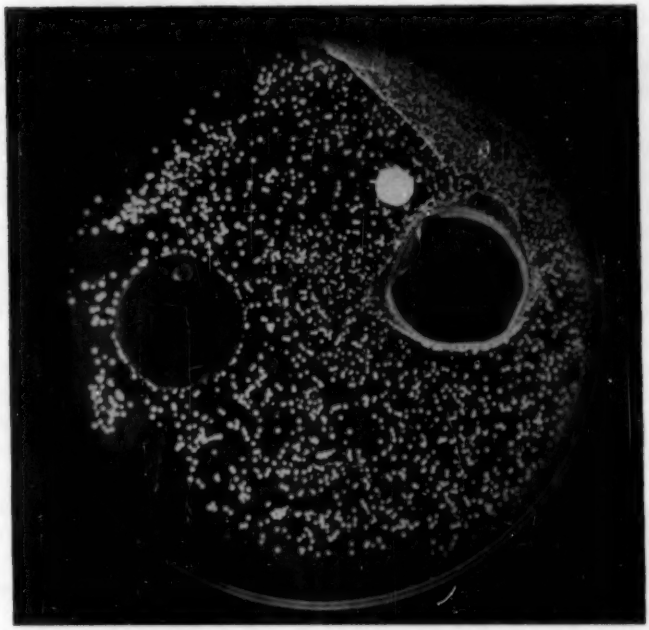

FIG. 1.—Lysis by γ phage of a culture consisting of S and Sm forms of *B. anthracis* grown on serum agar in CO_2. Plate showing isolated colonies used in order to demonstrate colony types.

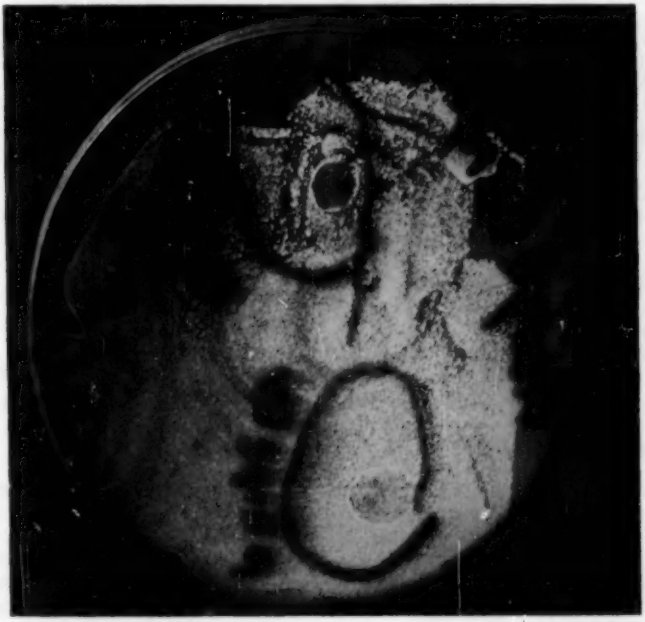

FIG. 2.—Top: lysis of R and RS forms of *B. anthracis* by γ phage. Bottom: failure of W phage to lyse these forms.

previously described were employed.

The photomicrographs, figures 1 and 2, show 18-hour cultures of *B. anthracis* 1014 in the S, R, and RS stages and clear zones due to the action of concentrated γ phage. Figure 1 shows typical smooth colonies which were grown on serum agar in CO_2 and clear zones indicating lysis by the γ phage. Figure 2 shows a plate on which both γ and W phage have been inoculated. The clear zone is indicative of lysis of the R and RS forms by the former, while the latter has little or no action on these forms.

DISCUSSION

Other workers have pointed out that the characteristics which distinguish *B. anthracis* from other bacilli, particularly *B. cereus*, are very difficult to define. Pathogenicity, notility, and ability to attack salicin are tests which are used to separate these organisms. These tests become less valid when one observes occasional motile *B. anthracis* strains[5,10,11] and nonmotile variants of *B. cereus*,[5,11] *B. cereus* strains virulent for mice[5,11,12] and nonpathogenic cultures derived from *B. anthracis*, and strains of both *B. cereus* and *B. anthracis* which vary in their nutritional requirements.[5,11,13] Smith et al[5] concluded that anthrax bacilli are nonmotile, pathogenic variants of *B. cereus*. Cowles[1] in 1931 and McCloy[2] later showed that certain bacteriophages, which they isolated, have a high species specificity which aids in distinguishing *B. anthracis* from the nonpathogenic bacilli. However, the phages which they studied either were not tested for ability to lyse variant forms of *B. anthracis* or had little or no action on them. The variant γ phage which is described in this paper has been shown to lyse not only the S and Sm variants of *B. anthracis* but also all other variants which could be isolated and tested. McCloy stated the the presence of a capsule on cultures incubated either in air or in CO_2 on serum agar prevented or inhibited lyis by the W phage. In contrast, the γ phage studied by the authors lysed the Sm (encapsulated) forms of *B. anthracis* when tested under similar conditions. During the course of the work reported in this paper, the γ phage lysed all forms of the anthrax organisms and failed to lyse any culture of any other species of the genus *Bacillus* which was tested. In comparison, only the R form of *B. anthracis* was susceptible to the W phage, while two cultures of *B. cereus* also were lysed. The reports of Tomcsik which showed that specific polysaccharide septa make up the framework of the anthrax capsule may help to explain the mechanism of the specificity of γ phage for the anthrax bacillus.[14,15]

The results of the present studies support the statements of McCloy[2] in regard to the value of phage tests in the

10. Bartley, E. D. 1930, The dissociation of *Bacillus anthracis*. M. D. Thesis, Belfast.
11. Nordberg, B. 1951, Studies of *Bacillus anthracis* in regard to its properties of diagnostic and pathogenic importance. Royal Vet. Col. Sweden, Stockholm, 1951.
12. Nungester, W. J. 1929, Carbohydrate fermentation, nitrogenous metabolism and catalase production by variants of *B. anthracis*, J. Infect. Dis. **46**: 214–224.
13. Sterne, M. 1937, The effects of different carbon dioxide concentrations on the growth of virulent anthrax strains. Pathogenicity and immunity tests on guinea pigs and sheep with anthrax variants derived from virulent strains, Onderstepoort J. Vet. Sc. & Animal Ind. **9**: 49–67.
14. Tomcsik, J. 1951, Complex structure of the bacterial capsule in the genus *Bacillus*, Experientia, **7**: 459–463.
15. Tomcsik, J. and Guex-Holzer, S. 1951, Anthrax polypeptid und andere spezies-spezifische substanzen der kapsel in der Bazillus-Gruppe. Schweiz. Zs. allg. Path. Bakt. **14**: 515–522.

differentiation of *B. anthracis* and *B. cereus*. Since phage tests are quite specific and can be read and interpreted usually within a period of 6 to 8 hours, their value as a tool for the rapid identification of *B. anthracis* becomes apparent.

SUMMARY

A variant bacteriophage (γ) which exclusively attacks all strains of *Bacillus anthracis* which have been tested is described. It differs from the parent W phage in that it is apparently capable of lysing all variant forms of strains of the anthrax organism. Some strains of *B. anthracis* which were resistant or only slightly susceptible to the W phage were attacked strongly by the γ phage under the conditions of these experiments.

RESIDUAL SURFACE DISINFECTION

I. THE PROLONGED GERMICIDAL ACTION OF DRIED SURFACES TREATED WITH ORTHOPHENYLPHENOL

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The failure of the available techniques for aerial disinfection to destroy pathogenic agents present on environmental surfaces and large dust particles has been demonstrated on numerous occasions. This defect has been a possible explanation for some of the disappointing results obtained in the use of ultraviolet irradiation and triethylene glycol vapor in field studies, and focussed our attention upon the need for more effective techniques of disinfection of environmental surfaces and the dust reservoirs. In line with this reasoning, our efforts were directed toward the search for a germicidal compound which would produce a persisting disinfectant action on treated surfaces in the dried state following application and which would be simple to use routinely. The potentialities of orthophenylphenol in this respect was brought to our attention by the work of Klarmann and his associates,[1] in which it was reported that surfaces treated with this compound in the form of O-Syl* had a residual germicidal action for organisms deposited upon them in the dried state. This report summarizes our laboratory studies with this compound and discusses their implication.

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1. Klarmann, E. G., Wright, E. S. and Shternov, V. A. 1951, In vitro studies relevant to control of secondary reservoirs of respiratory pathogens. Am. J. Pharm. **123**: 42–59.

* Orthophenylphenol is an odorless, water insoluble compound which, however, when incorporated with the various constituents included in O-Syl becomes capable of dispersion in the aqueous phase. The composition of O-Syl is as follows: orthophenylphenol, 12%; potassium ricinoleate, 25.8%; ethyl alcohol (denatured), 7.2%; propylene glycol, 7.5%; glycerol, 2.5%; water, 45%. Manufactured by Lehn and Fink Products Corporation, Bloomfield, New Jersey.

2. Dunklin, E. W. and Puck, T. T. A spray-drying technique for the preservation of bacterial cultures. To be published.

MATERIAL AND METHODS

For the purpose of this study, surfaces washed with dilute O-Syl solutions and allowed to dry were tested for their residual bactericidal action against dried bacterial particles. This was done under conditions whereby the attainment of bacteriostatic concentrations of orthophenylphenol would be prevented in the culturing process. Hence the changes in bacterial population observed could be attributed solely to the residual bactericidal action of the O-Syl treated surface and not be a result of the carry-over of the active germicidal material into the culture medium, with subsequent inhibition of growth.

The microorganisms used were derived from two sources:

(a) Dried powders containing either type I pneumococci or group C hemolytic streptococci obtained by spray-drying serum dextrose Difco heart infusion broth cultures after 7½ hours of incubation.[2] These spray-dried bacterial powders had a viable population of 10^{11} organisms per gram dry weight, and microscopic examination revealed that no particles greater than 5 μ in diameter were present.

(b) Those organisms found in ordinary room dust collected from vacuum cleaner bags, floor sweepings, and dusty shelves. The dust obtained was freed of fibers and large aggregates by passing it through a no. 200 sieve. The organisms found to be present in the sieved room dust consisted

TABLE 1.—*Amount of O-Syl residue retained by various materials after immersion in a 2.5% O-Syl solution and drying at 22 C at a relative humidity of 16%.*

Material	Mg of residue per g of material
Wood: one-half tongue blade	51.4
Wool blanket, 3″×1″ strip	41.0
Cotton sheeting, 3″×1″ strip	4.9
Glass: one-half microscope slide	0.2

of the saprophytic bacteria normally occurring in the environment, and at no time were any pathogens recovered from this material.

The surfaces upon which these dusts and powders were deposited consisted of glass, wood, wool and cotton. The glass surfaces were obtained by cutting standard microscope slides in half. The wooden surfaces were made by cutting tongue blades into 3 inch lengths, and the fabrics consisted of 3×1 inch strips of woolen blanket or cotton sheeting.

The glass, cotton and wool segments were washed with soap and water, rinsed thoroughly in distilled water, immersed in 70% ethyl alcohol for 1 hour, and then dried in sterile air for 1 hour. At this point, those specimens which were to be used for the germicidal testing were dipped into the desired O-Syl dilution in water and allowed to dry again. In the case of the wood strips, all specimens were sterilized in 70% ethyl alcohol and dried. The wood strips used in the test were then dipped in the desired O-Syl concentration and again allowed to dry.

When the standard glass surfaces were dipped into a solution of 2.5% O-Syl in distilled water and then allowed to dry in air of 10 to 20% relative humidity at 22 C, it was found that 2.2×10^{-4} g of dry residue per g of glass were retained on the surface. Since the other materials were much more porous, the amounts of O-Syl residue retained by them were proportionately greater, and these values are given in table 1.

TABLE 2.—*Bacterial counts obtained from glass surfaces inoculated with spray-dried pneumococci by various methods.*

A Swab counts	B Immersion count	Total A+B	Mean total count
	A. Spreading with spatula		
1070	335	1405	
970	730	1700	1465 ± 107
1137	225	1362	
1207	187	1394	
	B. Spraying and settling		
957	308	1265	
823	496	1319	
907	347	1254	1276 ± 28
684	553	1237	
887	416	1303	

In these experiments, two techniques were used to inoculate the control and test surfaces with either the spray-dried bacterial powders or the naturally contaminated dusts. In the initial experiments done with the glass surfaces, a small amount of the spray-dried bacterial powder was spread upon the surface with a spatula and the excess scraped off with a razor blade, leaving a thin film on the slide. As can be seen in table 2, this technique gave surprisingly close agreement among the individual inoculums as determined experimentally and was considered satisfactory although time consuming.

The second technique was used in the later glass and all of the wood and fabric tests. In this procedure, a small 60 liter chamber was used[3] and the test and control surfaces were placed on its floor and covered by a piece of cardboard arranged in such a manner that it could be raised to expose the specimens. The spray-dried bacterial powders or the sieved room dust were dispersed into the air in the chamber from a 250 ml Erlenmeyer flask by compressed air.[4] A small fan inside the chamber agitated the air and produced uniform mixing of the dispersed powders or dusts. After spraying for 5 minutes the fan was turned off, the cover lifted from the test and control surfaces, and the dispersed particles allowed to settle over a 20-minute period. As shown in table 2, this settling technique gave rise to closer agreement than was obtained by manually spreading the inoculum, although in both cases the deviation in initial population was less than ±10%.

Immediately after inoculation by either technique, the surfaces, both test and control, were placed into sealed constant humidity jars[5] and stored at 22 C. In each series, relative humidities of 16, 33, 52 and 75% were used.

Periodically, test and control specimens were removed from the humidity jars and sampled for total bacterial population. Each glass or wood surface was carefully swabbed with a dry, sterile

3. Robertson, O. H., Bigg, E., Puck, T. T. and Miller, B. F. 1942, The bactericidal action of propylene glycol vapor on microorganisms suspended in air. J. Exper. Med. **75**: 593–610.
4. Robertson, O. H., Lester, W. and Smith, M. 1951, The lethal effect of triethylene glycol vapor on dried air-borne bacteria. Am. J. Hyg. **53**: 69–79.
5. Robertson, O. H., Dunklin, E. W., Puck, T. T. and Lester W. 1948. The influence of relative humidity on the survival of respiratory pathogens dispersed into the environment. T. A. Am. Physicians, **51**: 280–287.

TABLE 3.—*Comparison of the recovery of spray-dried pneumococci from 1% O-Syl-treated slides by two methods of sampling. Spray-dried powder atomized into the air of the 60 liter chamber and the bacteria-containing particles allowed to settle upon the treated slides, which were simultaneously exposed and immediately sampled.*

Swabbing and immersion			Immersion only
Swab count	Immersion count	Total bacteria	Total bacteria
2370	642	3012	2920
2480	524	3004	2920
2090	786	2876	3080
2630	298	2928	2840
Mean		2955 ± 53	2940 ± 70

cotton swab which was immediately immersed in 5 ml of sterile heart infusion broth, shaken vigorously for 5 minutes, and then aliquots were plated in 5% sheep's blood agar. The glass or wood squares, after swabbing, also were immersed individually in 5 ml of sterile heart infusion broth and aliquots plated. The plates were incubated for 48 hours at 37 C and the plate counts combined to determine the total number of viable bacteria recoverable from the surfaces. The dry swab technique was devised for the purpose of ensuring that no appreciable quantity of the germicidal agent could come in contact with the bacterial cells in liquid suspension and exert a bacteriostatic action independently from that occurring on the dry treated surfaces. The data summarized in table 3 indicate that this did not occur.

In the case of the wool and cotton strips, the material was first sampled by slowly moving it

TABLE 4.—*Lack of effect of O-Syl residue upon the recovery of viable spray-dried streptococci allowed to settle upon wool blanket strips and sampled within 5 minutes of the time of inoculation.*

Exp. no.	Number of bacteria recovered					
	0.0% O-Syl			2.5% O-Syl*		
	Bubbler	Immersion	Total	Bubbler	Immersion	Total
1	1460	1103	2563	1554	966	2520
2	1220	635	1855	1227	757	1984
3	1622	1272	2894	1556	1404	2960

* Blanket strips dipped in 2.5% solution of O-Syl and dried thoroughly before inoculation.

for 2 minutes over the intake of a Folin bubbler air sampler[6] operating at a flow rate of 1 cubic foot per minute. The piece of wool or cotton then

6. Lemon, H. 1943, A method for collection of bacteria from air and textiles. Pro. Soc. Exper. Biol. & Med. **54**: 298–301.

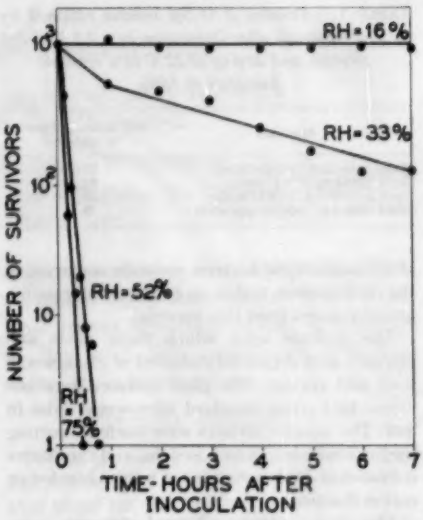

FIG. 1.—The survival of spray-dried pneumococci on glass surfaces treated with a 1% O-Syl solution and stored at various relative humidities.

was shaken vigorously for 5 minutes in sterile heart infusion broth. Aliquots were plated from the two samples, incubated and counted as before. As can be seen in table 4, there was good agreement between replicate samples when this technique was used.

EXPERIMENTAL RESULTS

Effect of relative humidity upon the rate of disinfectant action of O-Syl treated surfaces for spray-dried and dust-borne microorganisms.—When spray-dried pneumococci were deposited upon glass surfaces which had been prepared by immersion into a 1% O-Syl solution and allowed to dry, the results shown in figure 1 were obtained at various relative humidities. These data are summarized in figure 2, in which the rates of kill (K values measured in hours*)

* $K = \frac{\log N_0 - \log N_t}{t}$

where

N_0 = number of survivors at time 0

N_t = number of survivors at time t (in hours)

K_1 = initial rapid logarithmic death rate

K_2 = later, less rapid logarithmic death rate.

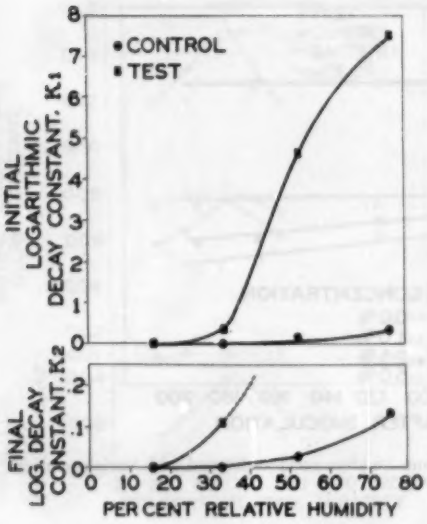

FIG. 2.—The effect of relative humidity on the survival of spray-dried pneumococci on glass surfaces treated with a 1% O-Syl solution.

are plotted as a function of the relative humidity. The bactericidal action of the O-Syl residue is immediately apparent at relative humidities above 33%. At 75% relative humidity, the bacteria on the O-Syl treated slides were killed at a rate twenty times faster than was found for the organisms deposited upon the control slides. At humidities above 33% no values could be obtained for K_2 since the initial rate of death (K_1) was so rapid no organisms survived to be measured. Similar data for spray-dried hemolytic streptococci deposited upon glass surfaces treated with a 5% O-Syl solution are summarized in table 5.

Thus, it appeared that minute concentrations of O-Syl residue remaining on glass surfaces were rapidly lethal to spray-dried bacteria originating from a broth suspension. However, it was important to determine the efficacy of O-Syl residue on glass surfaces against the more resistant naturally occurring, dust-borne microorganisms as a function of the relative humidity and the concentration of the germicide. The data obtained at 52% relative humidity and plotted semilogarithmically in figure 3 demonstrate the marked lethal effect of the O-Syl residue on dust-borne microorganisms. However, they also clearly indicate that at least several degrees of susceptibility to the germicidal action of the O-Syl residue exist among the organisms present in and upon the dust particles. This phenomenon is shown in the initial high rate of death (K_1), followed by a much slower lethal action (K_2), which is clearly seen in figure 3. The data for all relative humidities are summarized in table 6 and figures 4 and 7, in which it can be seen that the maximum lethal action of the O-Syl residue upon the dust-borne organisms occurred at 52% relative humidity.

Influence of concentration upon the rate of bactericidal action of O-Syl

TABLE 5.—*The bactericidal action of glass surfaces treated with a 5% O-Syl solution and inoculated with spray-dried hemolytic streptococci.*

Time of exposure, hours	Control—No O-Syl Relative humidity				Test—5.0% O-Syl Relative humidity			
	16%	33%	52%	75%	16%	33%	52%	75%
0	2940*	2940	2940	2940	2940	2940	2940	2940
0.5	3120	2830	2460	1700	2900	2480	60	3
1.0	3060	2940	2030	1040	2760	1940	1	0
1.5	2950	2230	1700	680	2840	1590	0	0
2.0	3000	3050	1750	560	2800	1390	0	0
4.0	3050	3160	1550	370	2560	640	0	0
6.0	2760	2850	1380	155	2600	250	0	0
8.0	3010	2780	1170	86	2320	95	0	0

* This is the average of 4 determinations: 2780, 2860, 3160, 2960.

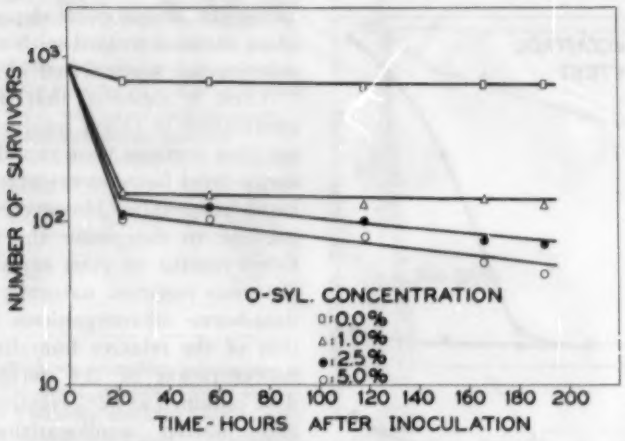

FIG. 3.—The survival of dust-borne microorganisms on glass surfaces treated with various concentrations of O-Syl. Relative humidity = 52%.

treated surfaces.—The rate of surface disinfectant action also is a function of the final concentration of O-Syl residue which is left upon the treated area after evaporation of the water has occurred. The data obtained suggest that there is a limiting concentration of the O-Syl residue beyond which the rate of bactericidal action does not increase significantly, and this would be expected. When a 1% solution of O-Syl was used to treat a glass surface at 75% relative humidity, a K_1 value of 7.6 was ob-

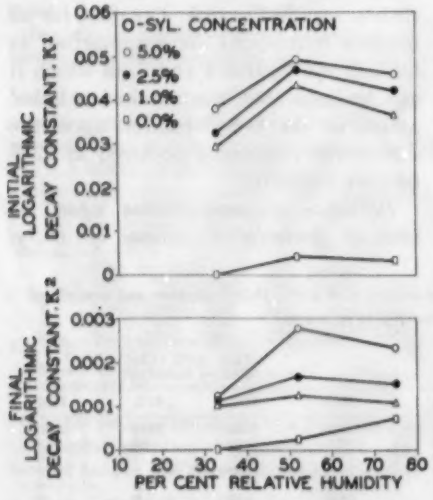

FIG. 4.—The effect of relative humidity and O-Syl concentration on the survival of dust-borne microorganisms on glass surfaces.

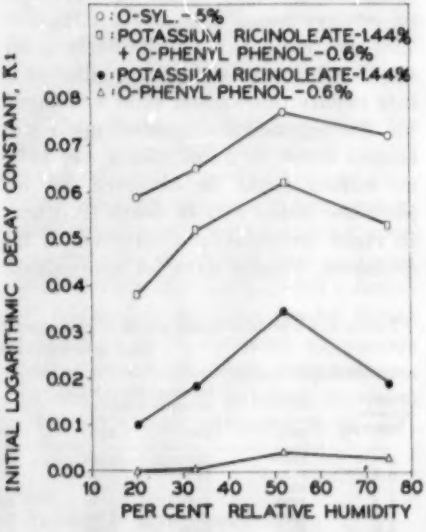

FIG. 5.—The rate of residual germicidal action of O-Syl and its individual components against dust-borne bacteria.

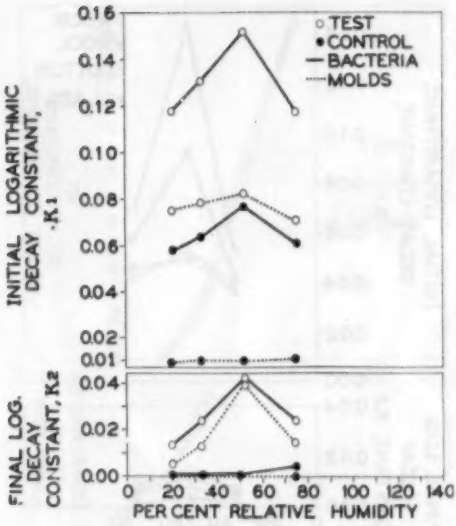

FIG. 6.—The effect of triethylene glycol vapor and relative humidity on the survival of dust-borne microorganisms on 5% O-Syl-treated glass surfaces.

tained for spray-dried pneumococci as compared to a value of 0.36 on the untreated control surface tested at the same humidity using a similar inoculum (fig. 2). However, when wood surfaces retaining approximately 100 times as much O-Syl residue as glass were tested under identical conditions, the maximum rate of disinfection gave a K_1 value slightly less than 15 (fig. 7). Thus, a 100-fold increase in concentration of O-Syl residue resulted in less than twice as rapid a rate of bactericidal action. This was shown even more clearly in figure 4, in which it can be seen that, in the case of the dust-borne organisms, 1% O-Syl treatment resulted in a 10-fold increase in the initial death rate (K_1) over that obtained on the untreated surface. Further increase in O-Syl concentration to 5% did not increase the initial rate of death by more than a factor of 1.1. However, even though the rates of disinfection of the microorganisms may rise only slightly with increases in the concentration of the germicidal residue above a certain minimal value, the numbers of bacteria recoverable will be significantly less at the higher levels.

It should be noted that the results obtained with the dust-borne organisms in relation to humidity are at variance with those obtained using spray-dried pneumococci. With the spray-dried pneumococci, the rate of disinfectant action of the O-Syl residue rose progressively as the humidity was increased and the maximum germicidal efficacy was obtained at 75% relative humidity rather than at 52%, as was the case for the dust-borne organisms.

Residual surface disinfectant action of the individual and combined constituents of O-Syl.—Orthophenylphenol is not water soluble and when deposited upon glass surfaces would remain a crystalline solid incapable of being absorbed by the dry bacterial cells. However, in the presence of the soap, potassium ricinoleate, contained in O-Syl, solution of the orthophenylphenol becomes possible. The additional ingredients, glycerol and propylene glycol, act as humectants assisting in the solubilization of the orthophenylphenol, and the combined action of these substances provides for the absorption of the active germicide by the bacterial cells. To confirm this hypothesis, experiments were carried

TABLE 6.—*The residual germicidal action of O-Syl against dust-borne microorganisms (2.5% O-Syl on glass surface).*

Time of exposure, hours	Control—No O-Syl Relative humidity			Test—2.5% O-Syl Relative humidity		
	33%	52%	75%	33%	52%	75%
0	896*	896	896	896	896	896
22	830	712	470	179	108	104
56	655	722	732	154	122	91
118	915	658	645	130	100	72
166	730	680	596	117	77	66
190	835	678	590	105	73	57

* This value is the average of three determinations: 945, 843, 900.

out using solutions of orthophenylphenol, potassium ricinoleate, combinations thereof and O-Syl. The solutions tested were made up to the concentrations of each substance present in a standard 5.0% O-Syl solution and their residual bactericidal actions determined.* Clean glass slides were dipped into these four solutions, air-dried and inoculated with dust-borne microorganisms in the 60 liter chamber. Slides coated with each of the test solutions were then placed in the constant humidity jars and sampled periodically. The results are given in figure 5, in which it can be seen that the water insoluble orthophenylphenol, when used alone, had practically no germicidal effect. Potassium ricinoleate alone had a definite bactericidal action. However, this action was almost doubled when orthophenylphenol was incorporated in the solution. The addition of the other constituents of O-Syl further increased the rate of death of the dust-borne bacteria. Thus, the presence of the soap, potassium ricinoleate, although itself possessing some germicidal action, causes the solubilization of the orthophenylphenol, thus making it available to the cell surface. The combined action of these two substances is further enhanced by the attraction of water molecules from the air by the hygroscopic glycerol and propylene glycol.

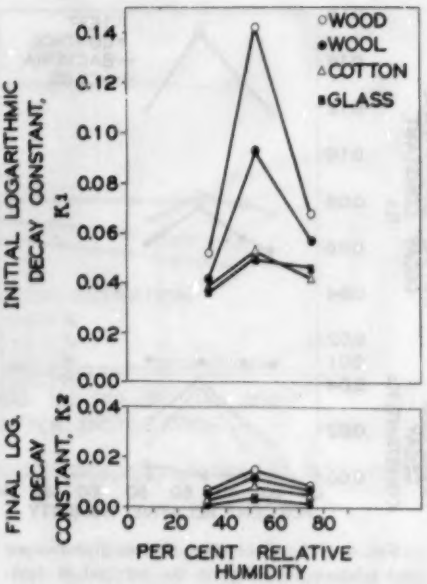

FIG. 7.—The effect of the type of material and relative humidity on the survival of dust-borne microorganisms on 5% O-Syl-treated surfaces.

Potentiating effect of triethylene glycol vapor upon the residual disinfectant action of O-Syl treated surfaces.—It has been observed on several occasions that triethylene glycol vapor was highly germicidal for freshly dispersed microorganisms atomized from broth cultures

* Solutions of the following composition were prepared: (1) orthophenylphenol, 0.6 g, acetone, 100 cc; (2) orthophenylphenol, 0.6 g, potassium ricinoleate, 1.44 g, distilled water, 98 cc; (3) potassium ricinoleate 1.44 g, distilled water, 98.5 cc; (4) O-Syl, 5 cc, distilled water 95 cc.

TABLE 7.—*The combined effect of triethylene glycol vapor and 5% O-Syl against dust-borne bacteria at various relative humidities. Glass surfaces used.*

Time of exposure, hours	Control—TEG* and no O-Syl Relative humidity				Test—TEG and 5% O-Syl Relative humidity			
	20%	33%	52%	75%	20%	33%	52%	75%
0	1558†	1558	1558	1558	1558	1558	1558	1558
14	245	164	130	160	50	32	13	50
30	142	122	76	87	32	12	4	13
38	130	134	74	77	20	9	2	8
58	151	119	64	64	12	2	0	2
72	138	125	70	64	8	1	0	1

* Atmosphere saturated with triethylene glycol vapor.
† This value is the average of 5 determinations: 1510, 1486, 1234, 1744, 1816.

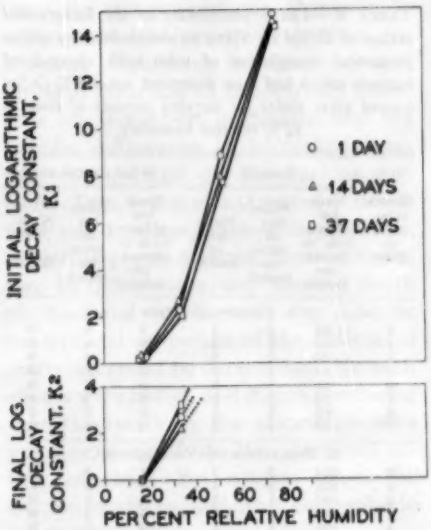

FIG. 8.—The undiminished germicidal activity of wool treated with a 5% solution of O-Syl and stored for periods up to 37 days.

but was relatively ineffective against the dried bacteria present in dust particles.[7–9] However, such air-borne bacteria-laden dust particles would become coated with condensed triethylene glycol when exposed to adequate vapor concentrations of this agent even though little or no germicidal action was demonstrated.[4] Therefore, it seemed quite possible that such glycol-coated dust particles would show an increased susceptibility to the germicidal action of O-Syl treated surfaces since the hygroscopic glycol film would enhance the absorption of the orthophenylphenol into the bacterial cells. To test this hypothesis, the air of the 60 liter chamber was saturated with triethylene glycol vapor originating from a warm beaker of the material. After a dense fog was apparent, the dust was dispersed into the chamber in the usual manner, allowed to settle upon the glass surfaces which then were placed in the constant humidity jars. In order to maintain a constant glycol saturation of the air in the jars, beakers containing aqueous glycol solution of such concentration that they would be in equilibrium with the various humidities were placed in the jars. The process of equilibration was accelerated by evacuating the jars to the partial presssure of water vapor corresponding to the desired relative humidity. Thus, the internal environment of each jar was kept saturated with triethylene glycol vapor at the desired humidity.

In these tests, molds (principally *Aspergillus niger*) and bacteria were counted separately and their respective survival rates determined. The results are given in table 7 and figure 6. It is evident that the germicidal activity of O-Syl treated surfaces is markedly enhanced by the maintenance of a saturated atmosphere of triethylene glycol vapor, as can be seen by comparison with the results shown in figure 4. Furthermore, it can be seen that the bacterial forms present in environmental dust are more readily destroyed by both agents than is the case with the molds.

In vivo irreversibility of the bactericidal action of O-Syl treated surfaces upon dried pathogenic microoganisms.—In an ef-

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fort to confirm the specific bactericidal action of the O-Syl residue upon pathogenic bacteria deposited upon the treated surfaces and the irreversibility of this process in vivo, infectivity studies using white mice were carried out. In these studies spray-dried pneumococci and streptococci were used. Glass slides were treated with a 5.0% O-Syl solution, dried and inoculated with the spray-dried bacteria by the settling technique previously described. The inoculated slides were stored in jars maintaining a constant relative humidity of 52%, and sample specimens were withdrawn at hourly intervals, shaken in 10 ml of sterile heart infusion broth, and aliquots of 0.5 ml injected intraperitoneally into white mice. Simultaneously, 0.5 ml aliquots also were plated in blood agar, and the resulting colonies counted after 48 hours incubation at 37 C, thus determining the approximate number of viable organisms inoculated into the mice. The results of these tests are summarized in table 8. It can be seen that all of the control mice died, whereas no deaths occurred when the injected bacteria had been exposed to the O-Syl treated surfaces prior to mouse inoculation. Pure cultures of the appropriate microorganisms, either pneumococci or streptococci, were obtained from the heart's blood of all of the mice which died. Thus, it seemed clear that all deaths were attributable to the intraperitoneal injection of the specific organism into the mice and that the fatalities were not related to nonspecific peritoneal contamination. These data indicate that the germicidal action of the O-Syl treated surfaces is not reversed in vivo and that the reduction in bacterial population determined by cultural techniques is directly correlated with the loss of ability to induce infection in the experimental animal.

TABLE 8.—*The irreversibility of the bactericidal action of O-Syl* in vivo, *as determined by intraperitoneal inoculation of mice with spray-dried bacteria which had been deposited upon 5% O-Syl treated glass slides for varying periods of time at 52% relative humidity.*

Hours exposure on slides	Control			O-Syl treated slides		
	Number of bacteria injected per mouse	Number of mice injected	Number of deaths	Number of bacteria injected per mouse*	Number of mice injected	Number of deaths
	A. Pneumococci (type I)					
0	64	5	5	3	5	0
1	44	5	5	0	5	0
2	39	5	5	0	5	0
3	43	5	5	0	5	0
4	27	5	5	0	5	0
5	14	5	5	0	5	0
6	10	5	5	0	5	0
7	10	5	5	0	5	0
	B. Hemolytic streptococci (group C)					
0	254	5	5	1	5	0
1	203	5	5	0	5	0
2	197	5	5	0	5	0
3	184	5	5	0	5	0
4	198	5	5	0	5	0
5	163	5	5	0	5	0

* This value represents the number of viable bacteria recoverable on culture from the slides. These slides were simultaneously exposed with those of the control series to the atomized spray-dried organisms and it is assumed that equal number of viable bacteria fell out upon both sets of slides. Therefore, the differences in viable organisms recovered would represent the germicidal activity of the O-Syl treated surfaces, and the discrepancy in numbers recoverable between test and control slides at any point in time would be accounted for by the number of nonviable organisms present upon the O-Syl treated slides.

Because of the possibility that the surfaces and bedding of a room might be important secondary reservoirs for aerial contamination and could be primary sources for contact infection, it was important to determine the germicidal efficacy of O-Syl residue upon materials other than glass. This was done by treating surfaces of wood, wool and cotton with 5% O-Syl solution, and, after drying, inoculating them with the bacteria-laden room dust by means of the settling technique. The results of these tests are summarized in figure 7, where it is seen that all of the surfaces tested were found to be bactericidal. Furthermore, reference to table 1 suggests that the degree of bactericidal activity determined experimentally is roughly correlated with the amounts of O-Syl residue retained and available

upon the various surfaces. However, in addition to the factor of retention, individual variations in chemical and physical structure of the various materials are important in determining the specific differences in bactericidal action encountered on the various surfaces. Two types of untreated controls were run in these tests—glass surfaces, as well as pieces of wood, wool and cotton. In this manner, any unusual death of bacteria attributable to specific bactericidal properties of the individual materials could be determined. No such effects were noted, and it was concluded that the death of the microorganisms was due primarily to the combined action of the O-Syl residue upon the treated surfaces and the atmospheric relative humidity.

Duration of residual disinfectant action action of O-Syl treated surfaces.—In all of the preceding experiments, the maximum elapsed time between the treatment of the surfaces with O-Syl and the inoculation with bacteria was 24 hours. Thus, it was necessary to determine the degree to which the treated surfaces retained this residual disinfectant action over longer periods of time. For this purpose strips of wool blanket treated with a 5% O-Syl solution were air-dried and stored at a relative humidity of 16%. Periodically, representative blanket strips were inoculated with spray-dried streptococci and deposited in constant humidity jars and the rates of bactericidal action determined as before. As can be seen in figure 8, storing the treated blanket strips for 37 days had no appreciable influence upon the rate of bactericidal action obtained at the various humidities.

DISCUSSION

During the past twenty years there has been an increased interest in the development of various techniques of aerial disinfection based upon the hope that the effective elimination of pathogenic agents from the air in crowded environments would lead to a reduction in the incidence of respiratory disease. Such techniques as ultraviolet irradiation and the vaporization of various germicidal compounds (propylene and triethylene glycol, hypochlorites, lactic acid and other α-hydroxy acids, hexylresorcinol, and I_2) have been developed on the basis of careful laboratory studies.[10–18] Of these techniques, only ultraviolet irradiation and glycol vaporization have been subjected to extensive field study.[7–9,19–22] From the re-

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sults obtained in the field studies it was apparent that these techniques, when properly applied, were capable of producing a significant reduction in the bacterial content of the air. However, they had no demonstrable effect upon the infectious particles deposited upon environmental surfaces or fabrics, and from these "secondary reservoirs" infection could be transmitted by means of direct contact or through redispersal of the pathogens into the air. In view of this fact various techniques were developed for the oiling of surfaces and fabrics so that the infectious particles would be mechanically retained and not be projected into the air following such activities as sweeping and bedmaking.[24–27] However, the various oiling techniques did not provide any enduring germicidal action on the treated surfaces and fabrics and hence permitted the infectious agents to survive and be capable of disease transmission through direct contact. When oiling was used in conjunction with air disinfecting techniques, maximal reduction of the bacterial population in the air occurred but this was not necessarily reflected in a decrease in the incidence of respiratory infections arising in the population inhabiting the treated environment.[8,19,22]

In an effort to provide a germicidal action superimposed upon the mechanical effect of oiling, various investigators incorporated disinfectant compounds in the oil-water emulsions which then were used to treat environmental surfaces and fabrics. Among the compounds used in conjunction with oiling were a cationic detergent, cetyl pyridinium bromide (Fixanol C) reported by Rountree[28,29] and a quaternary ammonium compound, alkyl-3-dimethylbenzyl ammonium chloride (Roccal), which was studed by Shechmeister and Greenspan.[30] In both instances, although a

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marked reduction in the bacterial contamination of the treated environmental surfaces occurred, no significant reduction in the incidence of cross infection was observed. Furthermore, the use of a disinfectant incorporated with the oil-water emulsion required an additional process in the treatment of blankets and bedclothes, which made routine use on a wide scale more difficult.

The next logical step was to develop a technique which would render the environmental surfaces actively germicidal for long periods of time without the need for additional processing and yet not be toxic, irritating, corrosive, or malodorous. In the search for such a technique various types of germicidal agents were tested without much success until the work of Klarmann[1] brought orthophenylphenol to our attention. This substance is the active ingredient of the commercial disinfectant O-Syl and was found to fulfill the above criteria satisfactorily.

From the data included in this report, it can be seen that when surfaces or fabrics have been treated with dilute solutions of O-Syl and allowed to dry, they become bactericidal and remain so for at least 1 month. This bactericidal action is due to the presence of orthophenylphenol in the residue, which is lethal to the microorganisms deposited upon the treated surface. Apparently there are several factors contributing to this residual surface germicidal action and, of these, the ambient relative humidity and the final concentration of O-Syl after evaporation are the most prominent.

The experiments described above demonstrate the pronounced effect of atmospheric relative humidity upon the rate at which the microorganisms were destroyed upon the treated surfaces. Two distinct patterns of interaction between the germicidal surfaces, relative humidity, and bacterial inoculums were observed. When spray-dried bacteria were used, prepared from rapidly growing broth cultures of pneumococci or streptococci, the rate of bactericidal action of the O-Syl residue increased progressively as the relative humidity was elevated above 33%. This suggested that, with spray-dried inoculums, the increasing water content available to the particles resulted in more effective penetration of the active germicidal component, orthophenylphenol, into the cells and, therefore, faster killing. In direct contrast to the above pattern, when O-Syl treated surfaces were tested against the mixed bacterial flora retained on dust particles, a maximum rate of surface disinfection was obtained at a relative humidity of 52%, and then the germicidal effect declined progressively as the water vapor content of the air was further increased. At the present time, no explanation can be given for these differences in the patterns of response to the interaction of relative humidity and the surface disinfectant action of residual O-Syl on the part of spray-dried bacterial cells and the naturally occurring organisms present in dust particles. Undoubtedly, variations in the metabolic and physical states of the two types of inoculums play a significant role in these patterns of response to these lethal stresses. It should be emphasized, however, that although these differences in patterns of response can be easily demonstrated, the rates of disinfection in all instances are sufficiently high at ordinary humidities (33% or above) that a progressive decline in the total bacterial contamination upon the environmental surfaces is assured.

The triethylene glycol vapor, condensing upon the bacteria-ladden particles, exerted a slight specific germicidal ac-

tion but in addition, served as a solvent which assisted in the penetration of the active disinfectant, orthophenylphenol, into the microorganisms, and hence augmented the lethal effect of the O-Syl. The rate of disinfectant action of the O-Syl treated surfaces at 52% relative humidity was more than doubled when the room dust experiments were carried out in a saturated atmosphere of triethylene glycol vapor. This synergistic action of triethylene glycol vapor and the O-Syl treated surfaces has the practical benefit of enhancing the combined bactericidal effects of these two techniques and suggests their potential usefulness in a program of total environment disinfection. Thus, the air-borne infectious particles would be most susceptible to the glycol vapor, whereas the pathogenic agents deposited upon the environmental surfaces would be effectively destroyed by the action of the O-Syl residue. Furthermore, the hygroscopic action of the condensed glycol vapor would increase the bactericidal efficacy of the O-Syl treated surfaces in the lower ranges of humidity.

The results of these experiments strongly suggest that proper application of O-Syl residue to the surfaces of a contaminated environment would lead to a significant reduction in the bacterial population deposited upon the treated surfaces. This residual disinfectant action would be persistent and would act to prevent a build-up in the bacterial contamination of the treated surfaces, which could well eliminate them as significant vectors in the transmission of disease. Under these circumstances a more definitive assessment of the respective roles of air-borne and direct person to person transmission of disease could be obtained. Furthermore, the demonstrated enhancement of the residual surface germicidal action of O-Syl residue by adequate concentrations of triethylene glycol vapor would provide more adequate environmental disinfection in the event these techniques were to be used.

SUMMARY

1. A new method for rendering treated environmental surfaces self-sterilizing is reported, and the techniques of testing its efficacy against dust-borne and dried bacteria are described in detail.

2. Dry glass surfaces, previously treated with dilute solutions of O-Syl, were found to be actively bactericidal against spray-dried group C hemolytic streptococci or type I pneumococci. This residual surface disinfectant action was found to be profoundly influenced by the ambient relative humidity, with the rate of germicidal action increasing as the humidity was raised.

3. Similar surfaces were likewise found to be actively germicidal against the microorganisms, both bacteria and molds, present in particles of natural room dust, although the rate of disinfectant action was significantly slower than that observed with spray-dried bacteria. Furthermore, the maximal rate of surface disinfection was found to occur at a relative humidity of 52% and decreased at levels above and below this value.

4. The primary active bactericidal component of the O-Syl residue was found to be the orthophenylphenol which, however, because of its insolubility, required the presence of potassium ricinoleate and humectants for effective surface disinfection. These additional constituents permit the solubilization of the orthophenylphenol with consequent penetration of the germicide into the bacterial cell.

5. The presence of saturation concentrations of triethylene glycol vapor

in the air markedly enhanced the germicidal activity of surfaces treated with O-Syl and rendered them effective at lower relative humidities than otherwise would be the case.

6. Animal experiments indicated that the disinfectant action of the O-Syl residue was germicidal rather than bacteriostatic and that this action was not reversible in vivo.

7. Effective germicidal concentrations of O-Syl residue were readily obtained upon wood, wool and cotton surfaces. These treated surfaces retained their residual disinfectant action essentially undiminished for periods up to 30 days after the initial application of the O-Syl.

THE EFFECT OF SURFACE ACTIVE AGENTS ON THE TOXICITY OF *PASTEURELLA PESTIS* LYSATES

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Many surface-active agents (SAA) are known to destroy or partially inactivate bacterial toxins[1] but none of the SAA has been reported to increase their toxicity. After it was demonstrated in this laboratory[2] that sodium desoxycholate increased the toxicity of filtrates of *Pasteurella pestis*, a study was undertaken to investigate the action of other SAA on these filtrates.

The purpose of this paper is to report the enhancing action of certain SAA on the bacteria-free filtrate obtained from an avirulent strain of *Past. pestis*.

METHODS AND MATERIALS

The toxin was prepared according to the method of Jawetz and Meyer.[3] Brain heart infusion agar (Difco) in Blake bottles was inoculated with a heavy suspension of an avirulent strain of *Past. pestis*, EV 76, and incubated at 37 C for 72 hours. The bacterial cells were harvested with a minimal amount of buffered saline,† pH 7.2, incubated at 37 C for 48 hours and kept at 4 C for 24 hours. The suspension was then centrifuged at 2000 r.p.m. for 90 minutes and the supernatant lysate filtered through Seitz or Berkefeld filters. The filtered lysate, containing the toxic products, will be referred to as the filtrate. The filtrate was plated for sterility and titrated for toxicity in 15 to 25 g white mice by the intra-abdominal injection of 0.5 ml of 4-fold dilutions. Groups of 5 mice were used for each dilution. The LD_{50} was calculated according to the method of Reed and Muench[4] and the titer was expressed as the reciprocal of the dilution killing 50% of the mice within a period of 10 days. The greatest number of deaths occurred within 2 to 12 hours after injection.

Several anionic, cationic and nonionized SAA were tested. A 1% solution of each compound (See table 1 for compounds and molarity used.) was prepared in sterile distilled water, added in equal volume to the filtrate, mixed, allowed to stand at room temperature for 15 minutes, serially diluted and injected into mice. The pH of the mixtures ranged from 6.9 to 7.1. Controls for the toxicity of the SAA were included with each experiment. Each SAA tested was titrated three times and the mean of the three titers was computed. Groups of 5 mice were used for each 4-fold dilution in the three titrations, which in effect amounted to 15 mice per group. The results were subjected to statistical analysis as indicated in table 2.

EXPERIMENTAL RESULTS

The results of the action of the SAA tested are summarized in table 2. Each SAA was tested for its toxicity for white mice by the injection of 0.5 ml of a 1:10 dilution of the 1% solution. Thus, the greatest concentration of the agent used was equivalent to 0.5 mg. All of the anionic compounds increased the toxicity by 3-fold to 4-fold amounts while none of the nonionized compounds affected the titer of the filtrate. Some of

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† 57 ml $M/15$ KH_2PO_4; 143 ml $M/15$ Na_2HPO_4; 1.7 g NaCl.

TABLE 1.—*The chemical composition, or type, of SAA tested with molecular weight and molarity used.*

SAA	Composition, or type	M.W.	Highest concentration used (in molarity)*
Anionic:			
Dreft	Sodium lauryl sulfate	288	0.003
Drene	Triethanolamine lauryl sulfate	324	0.003
Desoxycholate	Sodium desoxycholate	414	0.002
Orvus	Sodium lauryl sulfate	288	0.003
Tergitol 4	Sodium tetradecyl sulfate	316	0.003
Tergitol 7	Sodium alkyl sulfate	353	0.002
Cationic:			
Cetab	Cetyltrimethylammonium chloride	367	0.002
Emulsept	Lauric ester of colaminoformylmethylpyridium chloride	370	0.002
Hyamine 1622	Diisobutylcreoxyethoxyethyldimethylbenzylammonium chloride	466	0.002
Roccal	Mixture of alkyldimethylbenzylammonium chloride	?	(0.1%)
Triton K-60	Lauryldimethylbenzylammonium chloride	342	0.002
Nonionized:			
Carbowax 1500	Oleic acid ester of a polymerized polyethylene glycol	500	0.002
Triton A-20	Polyether alcohol	?	(0.1%)
Triton X-45	?	?	(0.1%)
Tween 20	Polyoxyalkylene ether of partial lauric acid ester	?	(0.1%)
Tween 60	Polyoxyalkylene ether of stearic acid ester	?	(0.1%)
Tween 80	Polyoxyethylene (20) sorbitan mono-oleate	?	(0.1%)

* The inoculum consisted of 0.5 ml of this solution.

the cationic agents increased the toxicity by 2-fold to 3-fold amounts while others had no effect. The enhancement of toxicity by anionic agents appeared to be greater than that produced by those cationic agents which were effective.

The increase in toxicity by the action of a SAA could be reversed by the addition of a second SAA to the mixture. For example, as seen in table 3, the increase in titer by desoxycholate from 25 to 160 was reversed to 20 by the addition of Triton K-60. In each case of such reversal, the titer reverted to approximately that of the filtrate alone. The action of a SAA on the filtrate was inhibited by mixing it with a second SAA before adding the filtrate. Thus, when two agents, Dreft (anionic) and Hyamine 1622 (cationic), were mixed before adding the filtrate no increase in toxicity occurred. Even when an active agent, desoxycholate (anionic) and an inactive agent, Triton K-60 (non-ionized) were mixed, no increase occurred when the filtrate was subsequently added to this mixture.

DISCUSSION

The toxicity for white mice of the *Past. pestis* toxin, when obtained as a crude filtrate, varied considerably even though prepared under similar environmental conditions. The titers of some preparations made in this laboratory ranged from 10 to 200. The titer of the crude filtrates used in this study averaged about 25.

The results show that the toxicity of

TABLE 2.—*Effect of SAA on the toxicity of the filtrate.*

Anionic SAA	Titer	Cationic SAA	Titer	Nonionized SAA	Titer
Drene	128*	Cetab	80*	Tween 20	28†
Dreft	120*	Roccal	80*	Triton X-45	28†
Desoxycholate	112*	Hyamine 1622	50†	Tween 60	25†
Orvus	109*	Triton K-60	24†	Tween 80	25†
Tergitol 4	94*	Emulsept	24†	Triton A-20	24†
Tergitol 7	94*			Carbowax 1500	24†

Filtrate with saline 32.

* Application of Chi square with Yates' correction showed a significant difference ($P < 0.01$) between the number of deaths in the experimental and control groups at comparable dosage levels.

† No significant difference between experimental and control groups.

TABLE 3.—*Interference of enhancement of filtrate toxicity by combined SAA.*

Primary mixture	Titer	Agent added to primary mixture	Titer
Filtrate-saline	25		
Desoxycholate-filtrate	160	Triton K-60	20
Triton K-60-filtrate	20	Desoxycholate	14
Triton K-60-desoxy-cholate	0	Filtrate	20
Filtrate-saline	32		
Roccal-filtrate	54	Triton K-60	32
Triton K-60-filtrate	32	Roccal	19
Filtrate-saline	17		
Hyamine 1622-filtrate	48	Dreft	20
Dreft-filtrate	126	Hyamine 1622	40
Hyamine 1622-Dreft	(1/5)*	Filtrate	32

* Mice receiving the combined SAA were sick and 1 out of 5 died on the third day.

the filtrate was enhanced by all of the anionic compounds tested and some of the cationic compounds but not by the nonionized compounds. The increase in toxicity could be reversed by the addition of a second SAA of prevented by mixing two SAA before adding the filtrate. The interference appeared to be independent of the type of SAA, since a nonionized compound could reverse the increase in toxicity produced by the action of an anionic or a cationic agent on the filtrate. In no case was any of the SAA tested found to destroy the toxicity of the filtrate. Numerous references cited in the review of SAA[1] indicate that SAA are destructive to toxins and enzymes. For example, the toxin of *Corynebacterium diphtheriae*, *Clostridium welchii* or *Clostridium tetani* is inactivated by bile salts, soaps, detergents and other SAA. Also, the enzymatic activity of amylase, lipase, pepsin, trypsin and phosphatase is inhibited by SAA.

The reason for an increase in the toxicity titer of the plague filtrate by SAA, in contrast to the destruction of other toxins, is not understood. The plague toxin differs from the soluble exotoxins cited and has not yet been fully defined so the chemical nature of the enhancement is not known. Speculations on the mode of action would include the possibilities of a combination between two substances or the splitting of one substance with the release of a more toxic product. The fact that the increase in toxicity is reversible might favor the idea of a loose combination between a toxic component in the filtrate and the SAA. Although the mode of enhancement is not known, the fact that the toxicity of plague lysates can be noticeably increased by contact with some SAA suggests that contact of plague vaccines with SAA should be avoided.

SUMMARY

Some anionic and cationic surface active agents enhance the toxicity of filtrates prepared from an avirulent strain of *Pasteurella pestis*. This is in contrast to the inactivation of other toxins by the same agents as reported in the literature.

PRODUCTION AND PROPERTIES OF A PENICILLIN-RESISTANT STRAIN OF FELINE PNEUMONITIS VIRUS

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Of the many properties of the agents of the psittacosis-lymphogranuloma group which set them apart from other infectious agents usually called viruses (Andrewes, 1952), one of the most interesting from a biochemical viewpoint is their susceptibility to almost all of the chemotherapeutic agents active against bacteria: sulfonamides, penicillin, aureomycin, terramycin and chloramphenicol (Eaton, 1950; Hurst, 1953). Unless the unlikely assumption is made that each of these drugs is capable of subtly modifying the metabolism of every type of virus-susceptible cell in every potential host in such a way as to prevent virus multiplication without interfering with normal cell metabolism, it must be assumed that the drugs act on the one common denominator of all the possible host cell-virus relationships, the virus itself. In other words, it is highly probable that chemotherapeutic agents inhibit the growth of the psittacosis-lymphogranuloma group of viruses just as they inhibit the growth of bacteria, by interfering with enzyme systems in the agents themselves.

Therefore, study of the mechanism of action of chemotherapeutic agents on the psittacosis group of viruses should yield information concerning the nature of the enzyme systems possessed by these agents. If the viruses could be made resistant to chemotherapeutic agents, this would be further, but not conclusive, evidence for a direct drug action on virus enzymes. In addition, the resistant virus might exhibit characteristics which would help to understand how the chemotherapeutic agent prevents virus multiplication. Golub (1948a) has demonstrated that drug resistance can be produced in the psittacosis-lymphogranuloma group by making psittacosis virus resistant to sulfadiazine.

This paper describes the production of a strain of feline pneumonitis virus highly resistant to penicillin by repeated chick embryo passage in the presence of the antibiotic and compares some of the properties of the parent and resistant virus in both the presence and absence of penicillin. Penicillin was chosen because of the comparative wealth of information concerning its action on bacteria (Work, 1952); feline pneumonitis virus, because of detailed studies on the effect of penicillin on the growth rate and morphology of the virus in chick embryos under a variety of conditions (Hamre and Rake, 1947; Eaton et al, 1948; Weiss, 1950).

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MATERIALS AND METHODS

Virus.—The strain of feline pneumonitis virus (Baker, 1944) used in these studies has been maintained for many years by continuous passage in chick embryos. Some of its general characteristics are described by Hilleman (1945) and Moulder and Weiss (1951)

Antibiotics.—The potassium salt of penicillin

G, aureomycin, terramycin and chloramphenicol were obtained from the usual commercial sources as dry, sterile preparations. Immediately before use, each antibiotic was dissolved and diluted in distilled water so that the desired dose for each embryo was contained in a volume of 0.2 ml. Many different penicillin G preparations gave identical results with both parent and resistant strains of virus.

Passage of virus in chick embryos.—Serial chick embryo passages of feline pneumonitis virus were made in the presence of penicillin to develop drug resistance and in its absence to determine the stability of the resistance so achieved. Fertile White Leghorn eggs from a single flock maintained on an antibiotic-free ration were incubated at 37 C for 6 days. The embryos were then inoculated via the yolk sac with 0.2 ml of a suitable dilution of the previous passage pool, usually 1:10. The inoculated embryos were reincubated at 37 C and candled daily. Deaths prior to the fourth day of infection were regarded as nonspecific.

Pools were prepared by placing 3 or more yolk sacs in a 200 ml centrifuge bottle containing glass beads and 2 ml of 0.1 *M* sodium monohydrogen phosphate buffer, pH 7.4, for each gram of yolk sac harvested. The yolk sacs were emulsified by shaking the bottles on a mechanical shaker for 15 minutes at room temperature. From each pool, several 1 ml aliquots were transferred to 10×150 mm Pyrex tubes, which were sealed in an oxygen-gas flame and stored at −60 C. The bacteriological sterility of each pool was tested by inoculation of thioglycollate broth. Duplicate pools were prepared to guard against loss of a passage by contamination.

For serial passage in the absence of penicillin, 5 or more embryos were inoculated with virus, and pools were prepared when the embryos died, usually on the 4th or 5th day of infection.

For passage in the presence of penicillin, 10 or more embryos were used. At least 6 embryos received penicillin and at least 4 received the virus alone. Penicillin was always administered via the yolk sac in a volume of 0.2 ml. The amount of penicillin given, the interval between injection of virus and penicillin, and the time the yolk sac pools were made all varied from passage to passage (table 1). The procedure finally adopted for serial passage of the highly-resistant virus consisted of giving 90,000 units of penicillin per embryo 1 hour after virus inoculation and harvesting yolk sacs from the first of the penicillin-treated embryos which died.

Titration of virus.—Details for titrating feline pneumonitis virus in chick embryos have been given by Moulder and Weiss (1951). Four 10-fold virus dilutions were injected into groups of 10 eggs. The LD_{50} was routinely obtained by the method of Reed and Muench (1938). If the dilutions employed for the initial titration did not bracket the 50% end point, the titration was repeated. Certain critical titrations such as those making up the growth curves of figure 6, were also calculated by the graphical logarithmic probit method of Miller and Tainter (1944).

Other pertinent experimental details are described in the following section.

RESULTS*

Effect of penicillin on the survival of embryos infected with parent and resistant virus.—It will be easier to understand the rationale of the method used to produce a strain of feline pneumonitis virus resistant to penicillin if the effect of the drug on the parent virus and the fully resistant strain obtained from it is first compared.

Figures 1 and 2 illustrate the effect of penicillin on the survival of chick embryos infected with PV. When penicillin was given 24 hours after virus inoculation (fig. 1), the average day of death of the embryos was prolonged by a period proportional to the logarithm of the penicillin concentration (Weiss, 1950), no doubt a reflection of the logarithmic rate of penicillin disappearance in fertile eggs (Hamre and Rake, 1947; Eaton et al, 1948). As little as 100 units per embryo protected over half of them for at least 10 days. Figure 2 demonstrates that when penicillin was given 48 hours after infection, it was much less effective in protecting the embryos

* The following abbreviations will often be used. The parent strain of feline pneumonitis virus from which the penicillin-resistant strain was derived will be referred to as PV (parent virus). The abbreviation RV will be used for the resistant virus. A specific passage of RV will be designated by RV followed by the passage number. For example, the virus obtained on the tenth chick embryo passage in the presence of penicillin will be called RV-10.

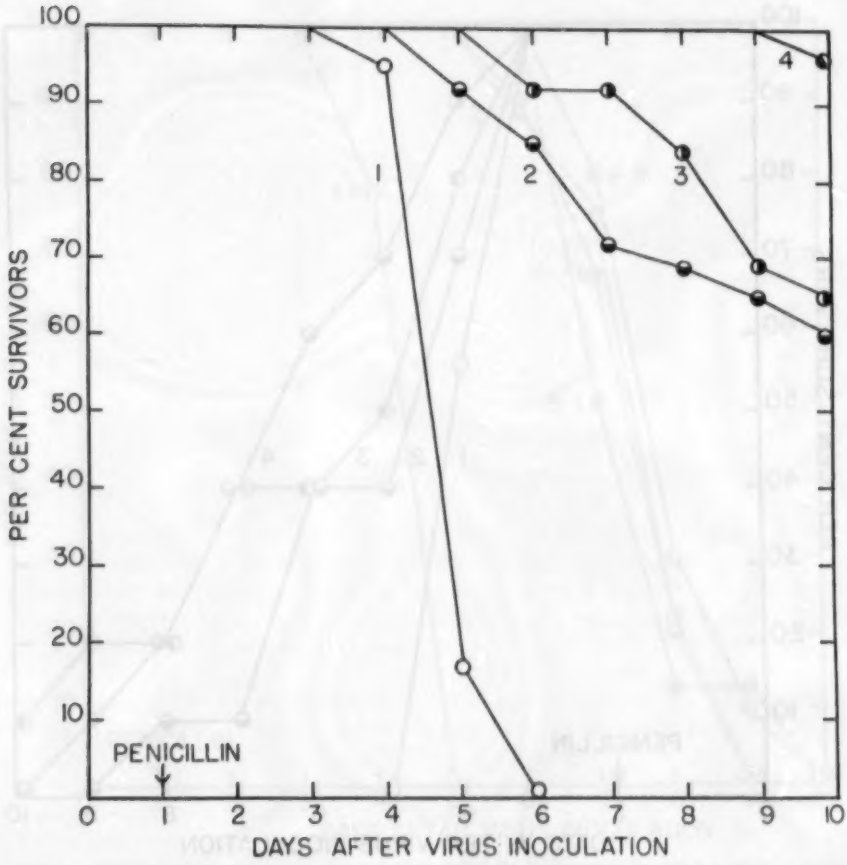


FIG. 1.—Survival of chick embryos receiving penicillin 24 hours after inoculation with parent virus. Six-day embryos were inoculated via the yolk sac with 10,000 LD_{50}'s of virus. Twenty-four hours later penicillin was administered as indicated below:

1. No penicillin
2. 100 units per embryo
3. 1000 units per embryo
4. 10,000 units per embryo

Twenty-five embryos were used for each penicillin level.

against PV. This diminished efficacy was used to advantage in the early passages in the presence of penicillin (table 1). By delaying administration of penicillin, larger amounts could be used and the danger of losing the passage strain was lessened.

The effect of penicillin on the survival of embryos inoculated with the highly resistant RV-40 is given in figure 3. From the similar survival times of embryos inoculated with either PV or RV-40, it appears that the two strains multiply at the same rate in the absence of penicillin. This conclusion is supported by more precise work (see fig. 6). The embryos given penicillin began to die about 2 days later than the ones

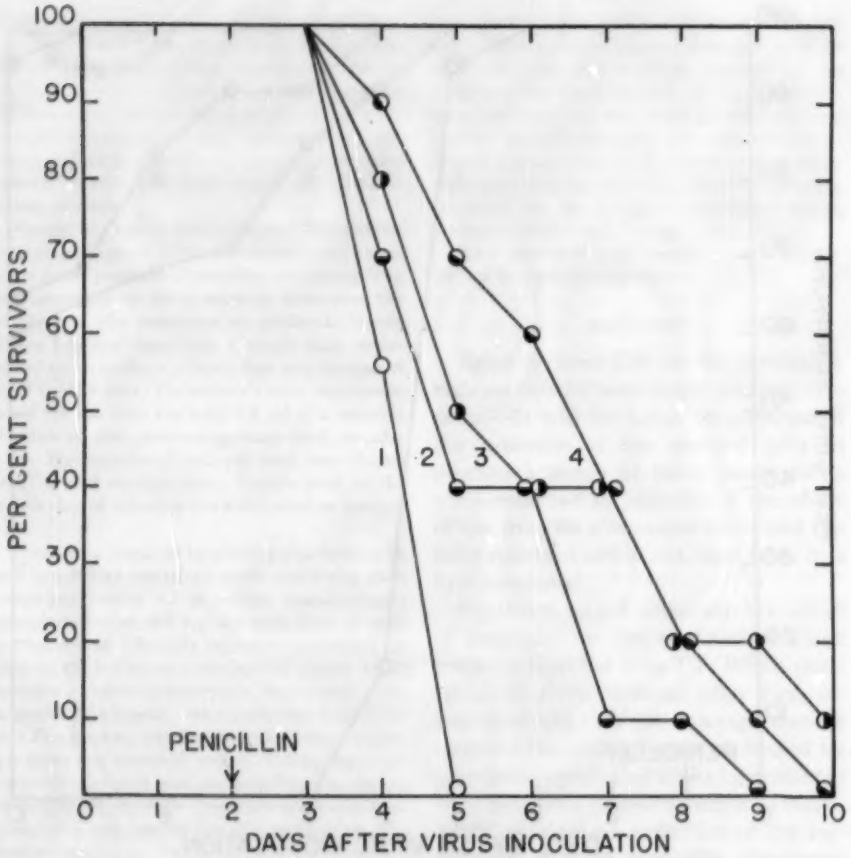


FIG. 2.—Survival of chick embryos receiving penicillin 48 hours after inoculation with parent virus. The experiment was performed exactly as described in figure 1, except that penicillin was injected 48 hours after infection.

receiving RV-40 alone, but deaths followed in rapid succession, and only very rarely did penicillin-protected embryos survive infection with the resistant virus. With RV-40, there is no relationship between protection and penicillin dosage. All amounts of penicillin from 100 to 100,000 units per egg produced almost exactly the same delay in death. Therefore, while RV-40 is not fully resistant to even 100 units of penicillin in the sense that embryos receiving 100 units do not die as fast as unprotected controls, it is fully resistant in the sense that no reasonable quantity of penicillin can keep the embryos from dying.

These properties of parent and penicillin-resistant feline pneumonitis virus allow the degree of resistance to the antibiotic exhibited by any passage strain, RV-*n*, to be determined by a very simple procedure. If 20 embryos are inoculated with RV-*n*, and after 24 hours each of 10 embryos receives 10,000 units of penicillin, the relative resistance can be estimated from the difference in

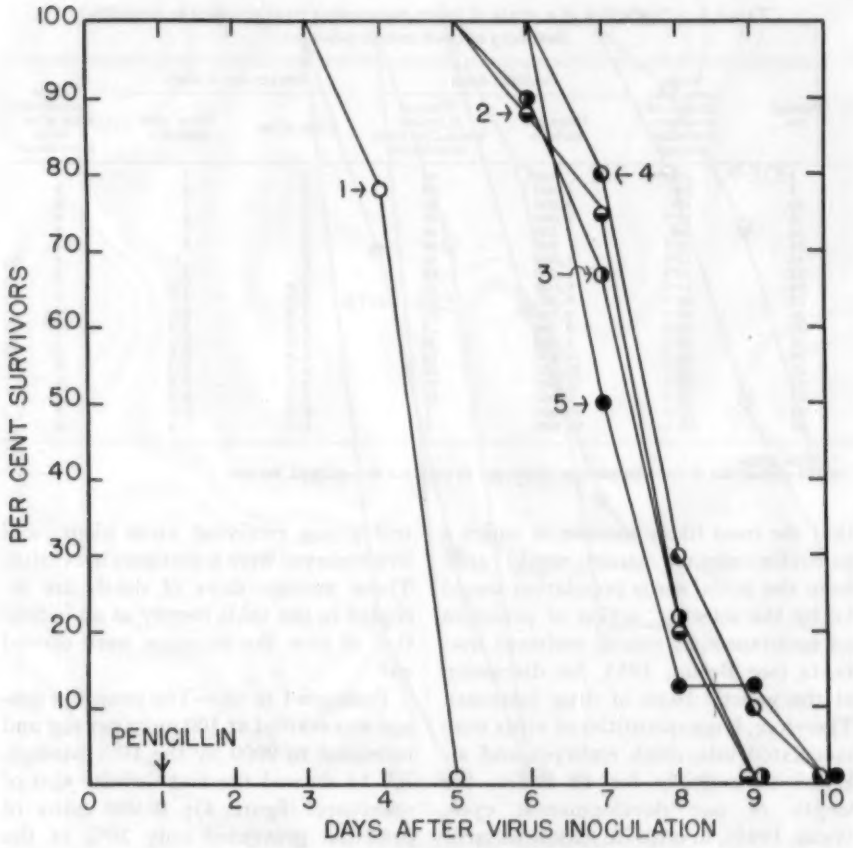


FIG. 3.—Survival of chick embryos receiving penicillin 24 hours after inoculation with resistant virus. The experiment was carried out as described in figure 1 except (1) the inoculum was RV-40 and (2) an additional level of penicillin was tested:

5. 100,000 units per embryo

average day of death for the two groups. Since, in the absence of antibiotic, the growth rates of PV and RV-*n* are the same, PV growth is represented by the death of the embryos receiving RV-*n* alone. In addition, it may be assumed that 10,000 units of penicillin will protect all of the embryos infected with PV for at least 10 days. Thus, it is not necessary to include in the test any embryos inoculated with PV. Finally, since all reasonable concentrations of penicillin greater than 100 units per egg produce the same lag in death of embryos injected with RV-*n*, only one penicillin concentration need be employed.

This simple assay of drug resistance was made an integral part of each penicillin passage subsequent to the 33rd, so that an estimation of the degree of penicillin-fastness is available for each of the last 14 passages (table 1)

Production of a strain of feline pneumonitis virus resistant to penicillin by continuous yolk sac passage in the presence of the antibiotic.—It was assumed

TABLE 1.—*Production of a strain of feline pneumonitis virus resistant to penicillin: Summary of chick embryo passages.*

Passage No.	Virus inoculum-dilution of pool from preceding passage	Penicillin dosage		Average day of death		Time of harvesting-days after virus inoculation
		Units per embryo	Time of injection-hours after virus inoculation	Virus alone	Virus plus penicillin	
1	10^{-9}*	100	48	5	7	7
5	10^{0}	5,000	48	4	5	5
10	10^{0}	9,000	48	5	7	8
15	10^{-1}	9,000	48	4	6	6
16	10^{0}	100	48	5	7	8
24	10^{-1}	900	48	5	5	5
25	10^{-1}	1,000	48	5	†	4
33	10^{-1}	1,000	48	5	5	5
34	10^{-1}	9,000	24	5.0	7.2	7
35	10^{-1}	9,000	24	5.0	7.7	7
36	10^{-1}	9,000	24	5.0	8.0	7
37	10^{-1}	9,000	24	5.0	7.8	7
38	10^{-1}	9,000	24	5.0	8.1	7
39	10^{-1}	18,000	24	4.8	8.6	7
40	10^{-1}	36,000	24	4.7	7.3	7
41	10^{-1}	72,000	24	4.4	7.8	5
42	10^{-1}	9,000	24	5.5	8.7	8
43	10^{-1}	90,000	1	6.0	8.4	8
47	10^{-1}	90,000	1	5.2	7.5	6

* Parent virus.
† The average day of death for passages 25 through 32 could not be calculated. See text.

that the most likely manner in which a penicillin-resistant strain would arise from the initial virus population would be by the selective action of penicillin on spontaneously arising resistant mutants (see Braun, 1953, for discussion of the genetic basis of drug fastness). Therefore, large quantities of virus were inoculated into chick embryos and allowed to multiply for 48 hours, the length of one developmental cycle Weiss, 1949), in order to expose as large a population as possible to the selective action of penicillin. The first few penicillin-treated embryos to die were used for preparing the inoculum for the next passage in the hope that they contained the highest proportion of penicillin-resistant mutants. As serial passage proceeded, the selective action of penicillin was gradually increased by raising the concentration of penicillin injected and by shortening the interval between virus inoculation and penicillin administration.

The average day of death given in table 1 for passages 1 through 33 is only approximate. Small numbers of eggs were often used, particularly in the control group receiving virus alone, and live embryos were sometimes harvested. These average days of death are included in the table merely as an indication of how the passages were carried out.

Passages 1 to 45.—The penicillin dosage was started at 100 units per egg and increased to 9000 by the 10th passage. RV-14 showed the first definite sign of resistance (figure 4); 10,000 units of penicillin prevented only 30% of the embryos infected with RV-14 from dying within 10 days.

Passages 16 to 24.—When RV-15 was passed at a level of 9000 units of penicillin per egg, no virus could be recovered. The penicillin dose was decreased to 100 units and then increased to 900 units by the 24th passage.

Passages 24 to 33.—In these passages, pools were prepared as early as possible in the belief that this might increase the proportion of resistant forms in the population. Living embryos inoculated with both virus and penicillin were sacrificed and their yolk sacs harvested as soon as the control embryos with virus alone began to die, or even 24 hours earlier, as

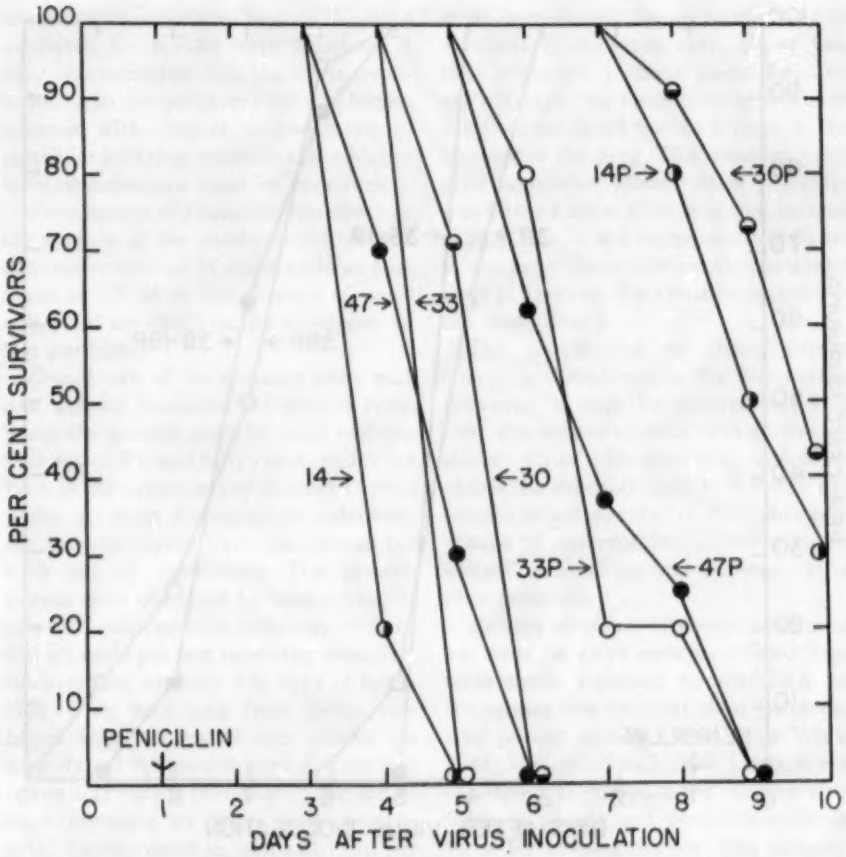


FIG. 4.—Penicillin resistance at different chick embryo passages. Twenty 6-day embryos were infected with 10,000 LD_{50}'s of each of the indicated passage strains. Twenty-four hours later, 10 received 10,000 units penicillin each. Survival curves of embryos receiving only virus are indicated by the passage number alone; those for embryos also receiving penicillin, by the passage number plus the suffix P.

predicted from results of the previous passage.

As shown in figure 4, RV-30 was no more resistant than RV-14. However, somewhere between the 30th and 33rd passage, there occurred a significant increase in penicillin resistance. When RV-33 was tested (fig. 4) for the first time in the serial passage, all embryos receiving virus and 10,000 units of penicillin died within 10 days of infection.

Passages 34 to 38.—Starting with passage 34, at least 10 embryos were always included in each group: those receiving only RV-*n* and those receiving both RV-*n* and penicillin. Only yolk sacs from dead embryos were harvested. Therefore, the degree of resistance shown by each passage strain could be estimated by the method outlined in the preceding section.

In passages 34 to 38, the selective

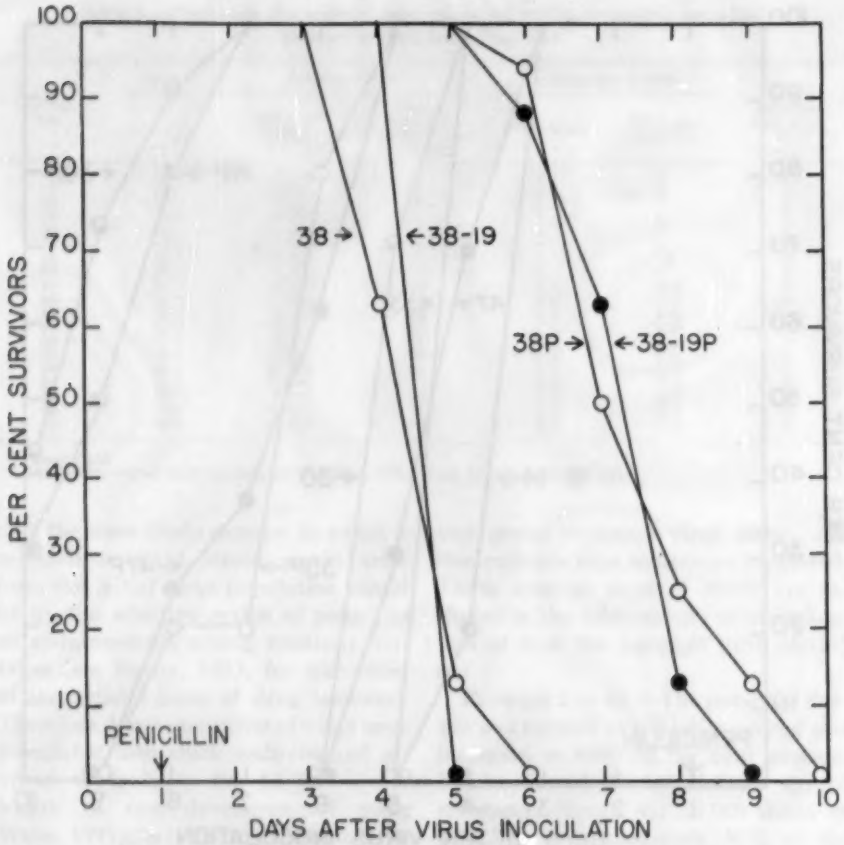


FIG. 5.—Permanency of penicillin resistance during chick embryo passage in the absence of penicillin. The experiment was conducted as described in figure 4. The same notation is employed. The following virus passages were tested:

RV-38

RV-38-19. RV-38 passed 19 times in chick embryos without penicillin

pressure of penicillin was increased by giving the antibiotic 24 hours after virus inoculation, instead of 48 hours as in previous passages, and great care was taken to prepare the inoculum for the next passage from the very first penicillin-treated embryos which died. Nevertheless, table 1 indicates that there was no further increase in resistance.

Passages 39 to 41.—The selective action of penicillin was further increased by raising the penicillin level in doubling increments from 9000 to 72,000 units per embryo. Again, penicillin resistance remained constant.

Passages 42 to 47.—Finally, the selective action of penicillin was further heightened by giving 90,000 units only 1 hour after virus inoculation, but this too failed to increase the degree of penicillin resistance. As shown in figure 4, the resistance of RV-33 and RV-47 was practically identical.

Since all these attempts to increase

the penicillin resistance beyond the level exhibited by RV-33 were fruitless, it may be concluded that the virus population from this point onward was homogeneous with respect to resistance to penicillin and that mutation to a higher level of resistance must be very rare.

Permanency of penicillin resistance in the absence of the antibiotic.—Figure 5 demonstrates that 19 chick embryo passages of RV-38 in the absence of penicillin had no effect on its resistance to the antibiotic.

Growth rate of the resistant virus with and without penicillin.—Figure 6 compares the growth rates in chick embryo yolk sac of PV and fully resistant RV-43 both in the presence and absence of penicillin. At least 3 completely independent growth curves were established for each set of conditions. The growth curves were obtained by inoculating 6-day-old embryos with sufficient virus to kill all embryos not receiving penicillin between the 4th and 5th days of infection. Five yolk sacs from living embryos were harvested and pooled on each day of the growth period. Care was taken to remove the entire yolk sac of each embryo, so that virus content could be expressed in terms of LD_{50} per yolk sac.

In the absence of penicillin, the growth rates of PV and RV-43 were identical, and the same maximum titer was reached on the 4th day, just prior to death of the embryos.

When 100,000 units of penicillin was injected into each egg 1 hour after virus inoculation, the virus concentration in PV-infected embryos remained at a low and unmeasurable level, indicating that effective inhibitory concentrations of the antibiotic were present throughout the period of observation. Similar results have been reported by Eaton et al (1948). However, in the presence of 100,000 units of penicillin, RV-43 almost immediately began to multiply at a relatively constant rate, about half that observed without penicillin, and after 9 days, the virus titer approached the titer achieved within 4 days in the absence of the drug. The resistant virus grew somewhat slower when penicillin was given 1 hour after infection instead of 24. Thus, in the experiments of figure 6, the penicillin-treated embryos died 5 days later than the controls instead of the usual 3 or 4.

The significance of these growth curves is considered in the *Discussion*. However, it may be pointed out here that the slower growth rate of the resistant virus with penicillin added explains the characteristic 3- or 4-day difference in average day of death between groups of embryos inoculated with resistant virus alone and resistant virus plus penicillin.

Relative virulence of parent and resistant virus for chick embryos.—Since bacteria made resistant to penicillin are sometimes less virulent than the sensitive parent strains (Work and Work, 1948; Miller and Bohnhoff, 1950), it was of interest to compare the relative virulence of parent and penicillin-resistant virus for chick embryos. The observation (fig. 6) that PV and RV-43 produced new infectious units at the same rate in the absence of penicillin suggested that there was no great difference in chick embryo virulence. However, the question was investigated more thoroughly.

Golub (1948b) has pointed out that in the psittacosis-lymphogranuloma group of viruses there exists a definite relationship between the size of the inoculum and the time required to kill chick embryos. When the logarithm of the number of LD_{50}'s inoculated is plotted against the average day of death, the resulting curve closely approximates a straight line. One measure of virulence

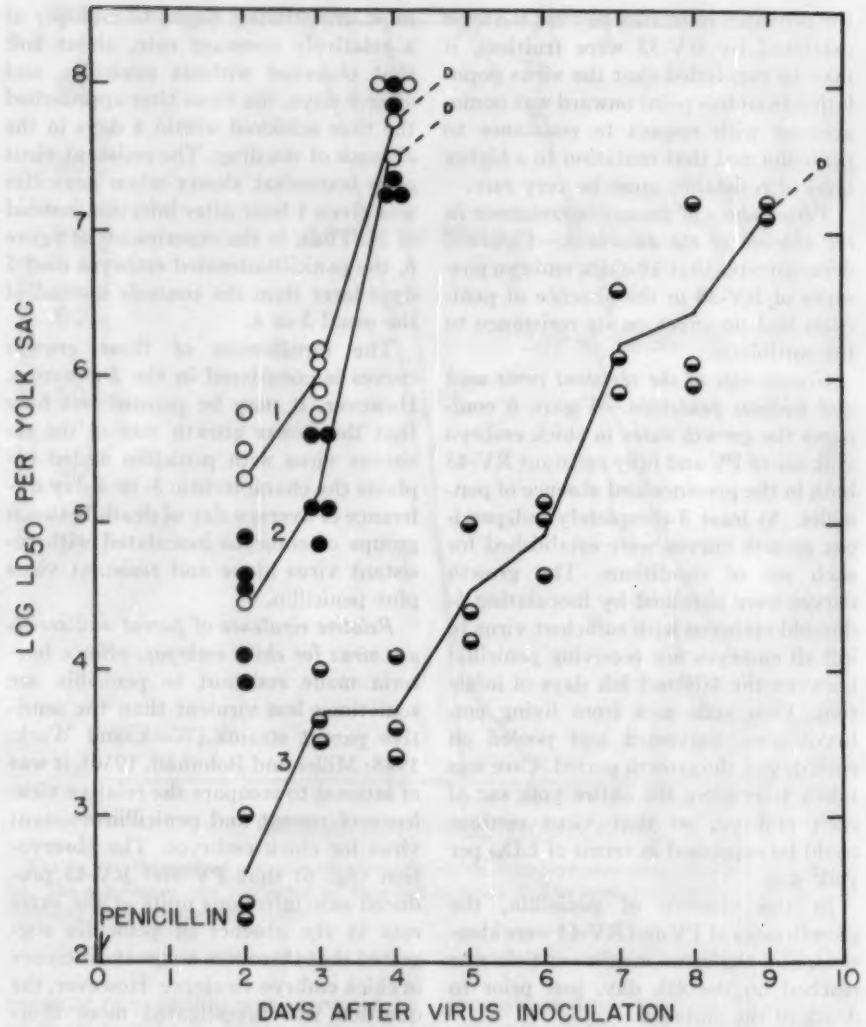


FIG. 6.—Growth rates of parent and resistant virus in the presence and absence of penicillin. Six-day embryos were inoculated with 100,000 LD_{50}'s of either PV or RV-43. One hour later, 10,000 units penicillin per embryo were given to half of them. See the text for further details.

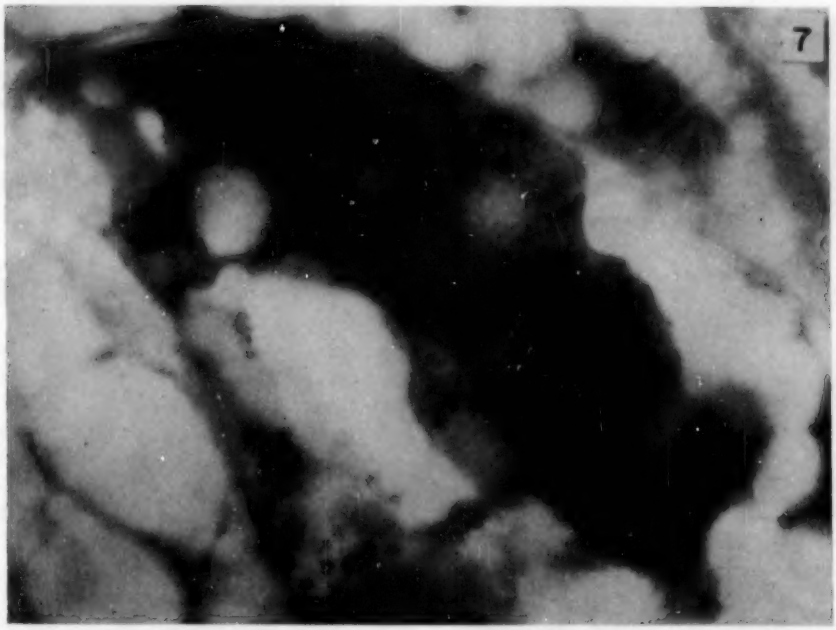
1. PV, no penicillin
2. RV-43, no penicillin
3. RV-43, penicillin

The virus titer in eggs receiving PV and penicillin was too low to measure.

Figures 7 through 15 are photomicrographs of parent and resistant virus growing in chick embryo yolk sac in the presence and absence of penicillin. Yolk sacs were embedded in celloidin, sectioned 10 μ thick, and stained with hematoxylin-eosin-azure-II. The photomicrographs were taken by Mr. Jean W. Crunelle of the University of Chicago Clinics. They are all reproduced at a magnification of 2000×.

FIG. 7.—A typical vesicle of the parent virus, 96 hours after virus inoculation. No penicillin.

FIG. 8.—A vesicle of RV-44, 96 hours after virus inoculation. No penicillin.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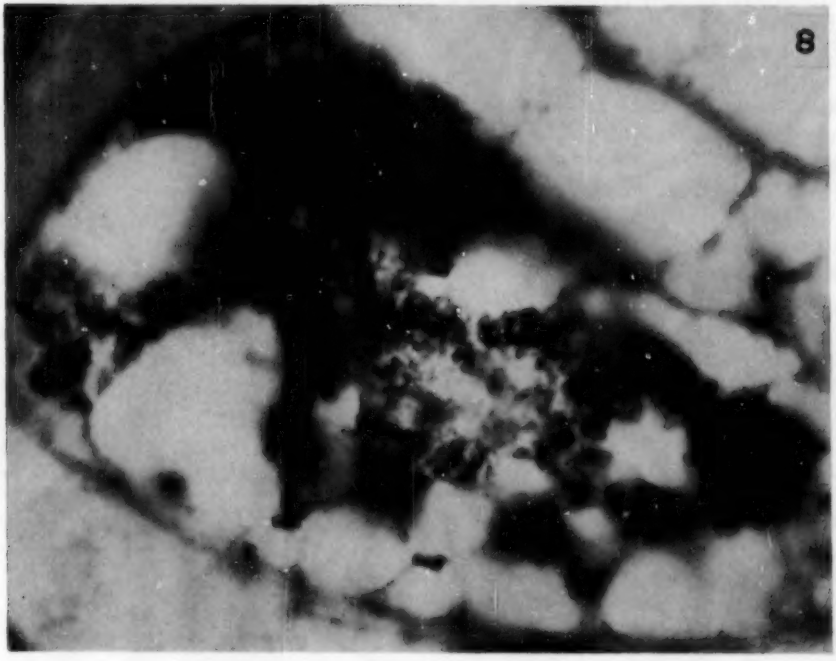
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TABLE 2.—*Comparison of the number of elementary bodies per chick embryo LD_{50} in parent and resistant virus.*

Virus	Stage of purification	Elementary bodies per LD_{50}
PV	After tryptic digestion	16.9
	After celite adsorption	15.4
	Mean of all determinations	16.2
	Standard error of mean	5.1
RV-44	After tryptic digestion	21.7
	After celite adsorption	27.0
	Mean of all determinations	24.3
	Standard error of mean	8.0

Three separate pools of both PV and RV-44 were purified and the chick embryo LD_{50} and elementary body count determined at each of the indicated stages in purification. Thus, each mean is derived from 6 determinations of the ratio of elementary bodies to LD_{50}'s. Student's t test indicates that the two means are not significantly different ($P = 0.4$).

in this group might then be the steepness of slope of such a line; i.e., the steeper the slope, the faster a given number of LD_{50}'s kills the embryo. When the log LD_{50} inoculated was plotted against average day of death for PV and RV-43, the straight lines fitted to the two sets of data by the method of least squares were identical. Their slopes were such that a 10-fold increase in inoculum size decreased the average day of death by about one day.

A more precise measure of the virulence of parent and resistant virus was obtained by determining the number of elementary bodies contained in one chick embryo LD_{50}. Large yolk sac pools of PV and RV-44 were subjected to the purification procedure of Zahler and Moulder (1953) in order to obtain preparations of elementary bodies of sufficient purity for counting by Gogolak's (1953a) method. Then, from the chick embryo titration and the elementary body count, the number of elementary bodies equivalent to 1 LD_{50} was calculated for each sample of virus (table 2). This method also indicates that development of penicillin resistance was not accompanied by loss of virulence for the chick embryo.

Relative virulence of parent and resistant virus for mice.—To determine mouse virulence, PV, RV-44 and RV-38-20 (the 20th penicillin-less chick embryo passage of RV-38) were established in mice by exposing them to an aerosol of 5 ml of a 1:10 dilution of heavily infected yolk sac emulsion in a cloud chamber described by Gogolak (1953b). The conditions of exposure were those recommended by him for murine pneumonitis virus. As was expected, none of these yolk sac-adapted strains killed mice or produced gross lung pathology on the first passage. However, on further passage of PV by exposing mice to a lung emulsion prepared according to Gogolak (1953a) and equivalent to the yield from 5 pairs of lungs, its virulence rapidly increased. On the 4th mouse passage, mice began to die on the 9th day of infection, and all mice sacrificed on the 10th day had fully consolidated lungs (table 3). In sharp contrast to PV, RV-44 and RV-38-20 showed no increase in virulence during the course of 4 mouse passages. On the 4th mouse passage, these strains killed no mice, and the lungs of mice sacrificed on the 13th day of infection had no gross macroscopic lesions and were of the same weight as the lungs of control mice exposed to an aerosol of the suspending medium alone (table 3). It is not possible to relate this decrease in mouse virulence to the development of penicillin resistance, since a similar change might have occurred in PV had it been passed in yolk sac an additional 44 or 58 (38+20) times without ever coming in contact with penicillin.

TABLE 3.—*Relative virulence of parent and resistant virus for mice on the fourth mouse passage.*

Virus	Day of infection		Weight of lungs from 10 mice (grams)
	First death occurred	Lungs harvested	
PV	9	10	6.8
RV-44	No deaths	13	2.8
RV-38-20	No deaths	13	2.9
Uninfected controls	No deaths	12	3.2

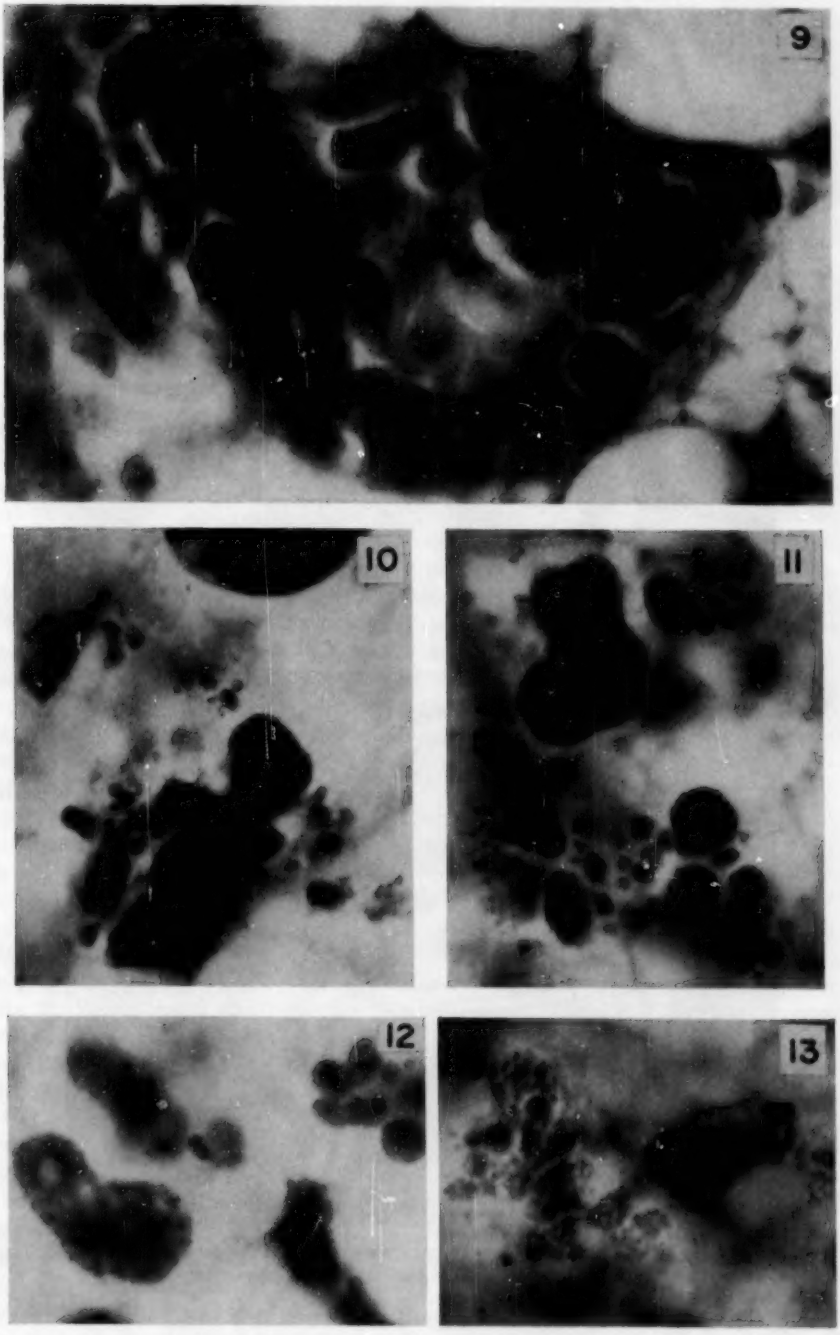
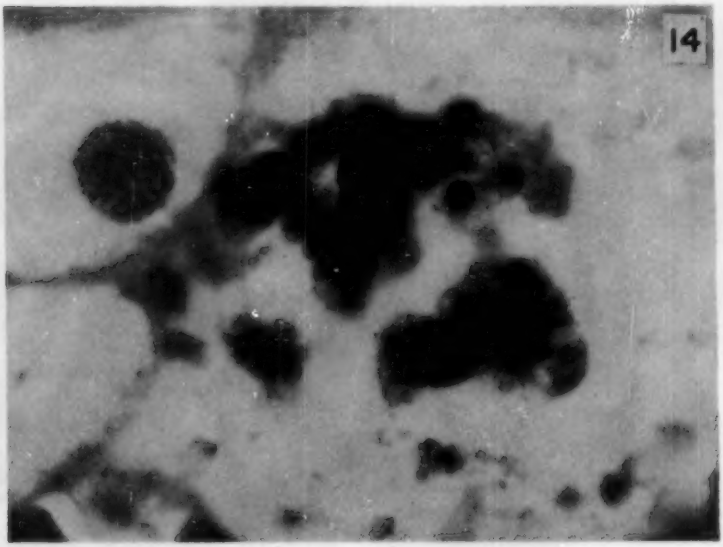


FIG. 9 through 13.—Vesicle structure of RV-44 in the absence of penicillin. Figure 13, 72 hours after virus inoculation; all others, 96 hours.



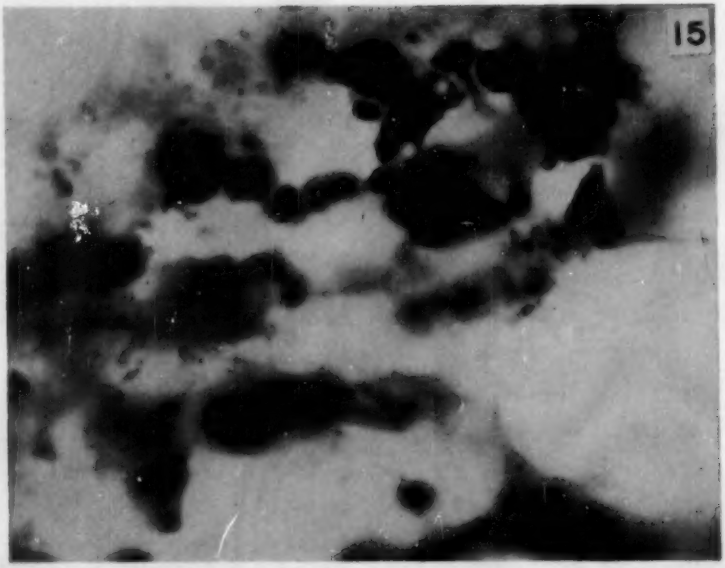


FIG. 14 and 15.—Vesicle structure of parent and resistant virus in the presence of penicillin. Ten-thousand units of penicillin were administered to each embryo 24 hours after infection.

FIG. 14—PV, 18 hours after penicillin injection.

FIG. 15.—RV-44, 18 hours after penicillin injection.

TABLE 4.—*Effect of chloramphenicol on parent and resistant virus.*

Virus	0.25 mg chloramphenicol per embryo			0.5 mg chloramphenicol per embryo		
	Number of experiments	Days life prolonged	Significance of difference between parent and resistant virus	Number of experiments	Days life prolonged	Significance of difference between parent and resistant virus
P		Mean ± S.E.	*P*		Mean ± S.E.	*P*
PV	7	1.84±0.16		7	3.27±0.15	
RV-38	4	2.13±0.11	0.2	4	3.08±0.46	0.7
RV-44	5	2.86±0.13	<0.01	5	5.20±0.24	<0.01

Six-day-old chick embryos were infected via the yolk sac with 10,000 LD_{50} of the appropriate virus, and 1 hour later chloramphenicol was injected by the same route. Each individual value for the prolongation of life by chloramphenicol was derived from the difference in average day of death between groups of 10 untreated and drug-treated infected embryos within a single experiment. To determine the significance of the differences between means, *t* and *P* were calculated from the difference between 2 means and the S.E. of the difference. Of 445 embryos receiving chloramphenicol, only 16 survived for more than 14 days after infection. These embryos were disregarded in calculating the average day of death.

Comparative resistance of parent and resistant virus to other antibiotics.—The susceptibility to aureomycin and terramycin of penicillin-resistant feline pneumonitis growing in chick embryo yolk sac appears identical to that of the parent virus.

Table 4 shows that RV-44 is more sensitive to the growth-inhibiting effects of chloramphenicol than PV. The heightened sensitivity is small but statistically significant. However, RV-38 is just as susceptible to chloramphenicol as PV, so the increased susceptibility to chloramphenicol must have arisen between the 38th and 44th penicillin passages, long after maximum resistance to penicillin had been reached. Therefore, the two alterations in drug reactivity cannot be directly related. Increased sensitivity to one agent during development of resistance to another has been observed in bacteria (Syzbalski and Bryson, 1952). It is usually explained by assuming that, during isolation of a strain resistant to the first agent, associated characters resulting in greater susceptibility to the second are also selected at the same time. The effectiveness of chloramphenicol against yolk sac infections of both parent and resistant feline pneumonitis virus is about the same as against similar infections with the viruses of psittacosis and lymphogranuloma (Smadel and Jackson, 1948; Wells and Finland, 1949).

Comparative morphology of parent and resistant virus.—The presence of penicillin in infected chick embryos causes striking changes in the morphology of the parent strain of feline pneumonitis virus (Weiss, 1950). To determine whether the acquisition of penicillin resistance is accompanied by morphological changes in the virus, yolk sac specimens of both parent and resistant strains, with and without penicillin, were taken for sectioning and staining.*

In the absence of penicillin, the vesicles of the parent virus are finely granular, homogeneous in texture, and tend to follow the protoplasmic strands of the infected cell (figure 8. See also, Weiss, 1949; 1950). Plaques are seldom found. RV-44 growing in the absence of penicillin shows considerable variation in its vesicle structure. Figures 9, 10, 11 and 12 show some commonly encountered types of vesicles. They contain many opaque plaques of varying size, similar to those found in vesicles of PV growing in the presence of penicillin. While most plaques reveal no interior structure, a few show a substructure composed of particles of various dimensions, the smallest of which approach the size of a normal elementary body

* We wish to thank Miss Alice Roberts for this work.

(fig. 12, 13 and 14). A small proportion of the vesicles of RV-44 are indistinguishable from those of PV.

RV-38-20, which maintained unchanged penicillin resistance through 20 drugless chick embryo passages (fig. 5), was morphologically indistinguishable from PV in the absence of penicillin. While it is difficult to state with certainty that the vesicle structure of RV-38-20 is entirely normal, there is no doubt that most of the abnormal morphological features of resistant virus were lost during the penicillin-less passages.

To compare the morphology of PV and RV-44 in the presence of penicillin, eggs were inoculated with both viruses and 24 hours later with 10,000 units of penicillin. Yolk sacs were taken for sectioning and staining at frequent intervals from 1 to 96 hours after penicillin administration. Penicillin produced similar morphological changes in both parent and resistant virus. As already described by Weiss (1950) for the parent virus, the effect of the antibiotic was visible within 2 hours in the form of enlarged particles within the virus clusters. Eight hours after penicillin inoculation, large dense bodies had formed. After 12 hours, many of these dense virus structures appeared to be embedded in a matrix of pale homogeneous virus substance (fig. 15 and 16). Large vacuolated plaques were commonly found in both parent and resistant vesicles. There was no observable difference in the morphology of PV and RV-44 through the entire 96-hour period of observation.

DISCUSSION

It is of interest to compare the action of penicillin on feline pneumonitis virus with its action on bacteria. Several points of comparison may be noted: (1) *Effective inhibitory concentration.*—Bacteria are generally regarded as penicillin-sensitive when their growth is inhibited by less than 0.1 unit per ml of medium. If 100 units of penicillin per egg are taken as the minimum inhibitory dose feline pneumonitis virus, then this amount evenly distributed in a 50 ml egg would represent a concentration of 2.0 unit per ml. However, since penicillin rapidly disappears from fertile eggs (Hamre and Rake, 1947; Eaton et al., 1948), the actual minimum effective concentration is probably much less. When fragments of yolk sac are removed from an infected embryo and incubated in vitro, only 0.1 unit of penicillin per ml in the suspending medium is required to prevent multiplication of feline pneumonitis virus (Moulder et al, 1953). (2) *Morphological changes.*—Since the original report of Gardner (1940), many investigators have found that bacteria produce enlarged, irregularly-shaped cells upon exposure to penicillin. Weiss (1950) concluded that the morphological changes induced by penicillin in feline pneumonitis virus are comparable to the changes observed in bacteria. (3) *Stepwise increase in resistance.*—When bacteria are serially transferred in mediums containing increasing concentrations of penicillin, drug resistance increases in a gradual, stepwise fashion (Demerec, 1948). Development of penicillin resistance in feline pneumonitis virus during serial passage in chick embryos occurred in at least 2 distinct steps (fig. 4). It is possible that still other steps were not detected by the relatively crude method used to measure resistance. (4) *Growth rate of resistant strains.*—Most strains of penicillin-resistant bacteria appear to grow more slowly than their parent sensitive strains (for example, see Bellamy and Klimek, 1948). In this respect penicillin-resistant feline pneumonitis virus differs from resistant bacteria, since it grows just as fast as the parent virus in the absence

of penicillin (fig. 6). (5) *Permanency of resistance.*—Penicillin-resistant feline pneumonitis virus shares with many penicillin-resistant bacteria the ability to maintain its drug fastness during repeated transfer in the absence of penicillin. Taken together, there is nothing in the various points of comparison to contradict the views that penicillin inhibits the growth of feline pneumonitis virus and bacteria in essentially the same way and that the penicillin-resistant forms of the virus are produced by the same mechanism operative in bacteria, the selection by penicillin of spontaneously arising mutants.

Lack of any real information concerning the reproductive mechanisms of feline pneumonitis virus prevents speculation about how penicillin inhibits virus growth or how the resistant virus is able to grow in the presence of high concentrations of the antibiotic. However, some tentative conclusions may be drawn concerning the general nature of resistant virus multiplication. It appears that the resistant virus still possesses the normal reproductive mechanisms of the parent strain, because the two viruses grow equally fast in the chick embryo in the absence of penicillin. In the presence of as little as 100 units of penicillin per embryo, the resistant virus may be assumed to grow by a mechanism completely unaffected by any amount of penicillin. The present data offer no clue as to what this mechanism might be. However, the penicillin-resistant growth process is relatively inefficient, and multiplication occurs at only one-half the normal rate. These concepts offer an explanation for the 3-day lag in death of embryos inoculated with resistant virus which may be produced by any quantity of penicillin from 100 to 100,000 units per egg.

Comparison of the experiments on growth rate and morphology of parent and resistant virus reveals two paradoxical situations. First, in the absence of penicillin, parent and resistant virus grow at the same rate, yet exhibit different morphological forms. Second, in the presence of penicillin, the two viruses grow at different rates, yet have the same morphological appearance.

The observation that RV-38-20 lost most of its aberrant morphology, but none of its antibiotic resistance, during penicillinless passage offers a possible resolution of the first paradox. It may be that penicillin resistance and abnormal vesicle structure are two independent characters which emerged during the course of the penicillin passages, the former relatively permanent and the latter lost in the absence of the drug. It appears that the altered morphological development shown by resistant virus upon first passage without penicillin does not affect the growth rate in the absence of the antibiotic and is not essential for exhibition of penicillin resistance.

SUMMARY

Serial yolk sac passage of feline pneumonitis virus in the presence of increasing penicillin G concentrations produced a strain of virus highly resistant to penicillin. Resistance reached a maximum in 33 passages and could not be further increased by 14 additional passages under a variety of conditions. At least one intermediate level of resistance was noted. Full penicillin resistance was maintained through 20 chick embryo passages without penicillin.

In the absence of penicillin, parent and resistant virus grew at the same rate. In the presence of sufficient penicillin to completely protect embryos inoculated with parent virus, the resistant virus grew at one half the normal rate and killed 100% of infected embryos about 3 days later than without the

antibiotic. In the range of 100 to 100,000 units per egg, the growth rate and lethality of resistant virus were independent of the penicillin concentration.

The resistant and parent virus were equally virulent for chick embryos in the absence of penicillin, but the resistant virus was much less virulent for mice.

Parent and resistant virus showed the same susceptibility to aureomycin and terramycin, but the resistant strain was more sensitive to inhibition by chloramphenicol.

The morphology of resistant virus growing in yolk sac without penicillin differed from that of parent virus and was characterized by the presence of many plaques similar to those produced by parent virus on contact with penicillin. The resistant virus lost most of its abnormal morphological appearance during 20 drugless passages. When penicillin was present, the morphology of the two strains appeared identical.

From these results and those of previous investigators, it was concluded that the growth-inhibiting effects of penicillin on feline pneumonitis virus resemble in several respects its effects on susceptible bacteria and that the penicillin-resistant virus was probably obtained through the selective action of the antibiotic on spontaneously arising resistant mutants.

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THE LYSOZYME SPECTRUM OF THE GRAM-NEGATIVE BACTERIA

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Fleming's[1] demonstration of the lysis of saprophytic air bacteria exposed to extracts of tissues and secretions of man containing lysozyme indicated that the enzyme is one of nature's defensive mechanisms. Lysozyme is currently associated with ulcerative conditions of the intestine. As a part of this syndrome, the role of lysozyme as it affects the ever-present gram-negative bacteria in the intestine is yet to be shown. The literature indicates general acceptance of the concept that these bacteria are not sensitive to the enzyme and therefore are not lysed. However, inhibition of growth and occasional lysis were observed with certain strains of pathogenic gram-negative bacteria when exposed to egg white, sputum, tears, or purified lysozyme.[1–7]

In 1952, Myerholtz and Hartsell[8] reported that gram-negative bacteria were, in general, not lysed when conventional methods were used. Only under special conditions could they observe that when the cells were exposed to lysozyme and then to the technique of Nakamura,[9] lysis would result. The effect of the enzyme was proportional to its concentration in the menstruum.

The present study was initiated to obtain the spectrum of lysozyme sensitivity of pathogenic and nonpathogenic gram-negative bacteria, and to test the effect of the enzyme on the viability of the species not lysed.

MATERIALS AND METHODS

Cultures.—One hundred and thirty-five organisms, representative strains of which are indicated in table 2, were obtained from several sources.*

Mediums.—The brucella strains were cultured on liver infusion agar (Difco). The neisseria and pasteurella strains were maintained on cystine trypticase agar (BBL). All other organisms were grown on yeast-water, veal-infusion agar.[10]

Procedure.—The organisms were cultured

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daily at 25 C onto their appropriate mediums twice prior to testing. Suspensions were prepared from 18-hour agar slants with 0.85% saline, filtered through cotton and then standardized to optical density (2-log G) 0.523 with a Coleman Universal Spectrophotometer Model-14. Lysozyme,* in twofold effective dilutions in double distilled water from 1:60,000 to 1:480,000, was added to the suspensions. Each dilution was then adjusted to pH 3.5 with $N/300$ hydrochloric acid. Each test mixture contained 2 ml of diluted lysozyme, 2 ml of cell suspension and 2 ml of the acid. Appropriate controls, with and without the enzyme and/or alkali, respectively, were also prepared.

The test suspensions were incubated at 45 C for 1 hour in a water bath. The addition of $N/20$ sodium hydroxide to give a final pH of 9.8 (the Nakamura technique) followed, before the temperature became lower than 40 C. Lysis, as indicated by clearing, was then determined spectrophotometrically before and after incubation and following the addition of alkali.

The lysis observed in the suspension tubes, after the addition of alkali, was considered as total lysis. That portion of the total clearing attributable to lysozyme action and that due to alkaline solubility factors were determined by comparison of the test suspensions with the appropriate control.

The total clearing (lysis) of the cell suspension was computed as the difference in optical density (OD) of the lysozyme-containing tubes before and after the hour of incubation and following the addition of sodium hydroxide. This value was calculated as percent of potential clearing, which was 100% for each species. The amount of clearing due to lysozyme was determined as the difference in clearing of the enzyme-containing tubes and the enzyme-free control tubes immediately following the addition of alkali. Lysis due to alkali was the difference between the total and lysozyme clearing. This change also included any autolysis that the cells may have undergone.

Viability studies.—To test for the viability of the species not lysed, the cells were prepared as above. Lysozyme was added to make final dilutions of 1:3,000 and 1:60,000. At pH 3.5 viability studies were meaningless because the acid had killed the cells. The pH of the respective tubes was then made 6.5 before incubation. In this series 2 ml of double-distilled water were substituted for the acid used in the studies of lysis. A lysozyme-free control was also tested. The number of surviving cells was determined by plate counts on yeast-water, veal-infusion agar after 0, 1, and 3 hours incubation and following the addition of sodium hydroxide. The plates were incubated at 37 C for 48 hours before counting.

* Lysozyme source: Armour and Company, Chicago 9, Illinois.

TABLE 1.—*Lysis of* S. bareilly *and* Pr. morganii *exposed to lysozyme and then the Nakamura technique.*

Organism	Lysozyme dilution $\times 10^3$				Control*
	1:60	1:120	1:240	1:480	
Initial density					
S. bareilly	0.201†	0.187	0.194	0.194	0.181
Pr. morganii	0.181	0.174	0.181	0.174	0.187
Density after incubation					
S. bareilly	0.215	0.215	0.215	0.215	0.208
Pr. morganii	0.194	0.187	0.187	0.187	0.194
Density after alkali					
S. bareilly	0.040	0.061	0.086	0.114	0.168
Pr. morganii	0.092	0.097	0.108	0.108	0.102

* Control contains cells, distilled water, pH 3.5.
† Readings in optical density (2-log G) against a distilled water blank adjusted to 0.000 density at a wave length 540 mμ.

EXPERIMENTAL RESULTS

Table 1 presents the results of a typical experiment. After the hour of incubation at 45 C the enzyme would appear to have had no effect on the cells of either species; however, after the addition of alkali both species were easily differentiated as to lysozyme sensitivity. The essentiality of the Nakamura technique for determining the effect of the exocellular enzyme, as well as autolytic enzymes, on the cell agrees with the conclusions of Grula and Hartsell,[11] Epstein and Chain,[12] and Ponomareva.[7] A direct ratio between the concentration of the enzyme and the extent of lysis was observed. This relationship was characteristic of all the organisms tested. The differences in clearing of the control tubes without lysozyme, subsequent to the hour of incubation and following the use of the Nakamura technique, are in sharp con-

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TABLE 2.—*Lysis of certain gram-negative species with lysozyme.**

Organism	Total lysis	Proportional changes due to:	
		Lysozyme	Alkali factors
Salmonella			
cholerasuis var. Kunsendorf	98†	77	21
pullorum #3	95	65	30
para-typhi B	92	62	30
bareilly	81	62	19
typhosa #59	83	37	46
gallinarum #469	65	32	33
Brucella			
suis #35	95	79	16
suis #1	88	73	15
abortus D	83	62	21
melitensis	73	44	29
bronchiseptica #139	72	44	28
Klebsiella			
pneumoniae 2M	88	55	33
pneumoniae 83B	87	51	36
pneumoniae BE PR	58	47	11
friedlander's bacillus	71	37	34
Shigella			
ceylonensis	82	67	15
para-dysenteriae #9	82	53	29
sonnei #1	88	45	43
alkalescens	90	43	47
dysenteriae #2	72	33	39
Neisseria			
catarrhalis	86	58	28
flavescens ATCC #9746	83	39	44
sicca ATCC #9913	88	37	51
perflava ATCC #10555	70	21	49
Pseudomonas			
pyocyanea	89	53	36
aeruginosa MSC	98	26	72
syncyanea U. Ch.	98	25	72
Pasteurella			
pseudotuberculosis ATCC #6902	90	57	33
multocida ATCC #9656	90	34	56
multocida ATCC #7773	80	33	47
multocida ATCC #6535	74	29	45
Erwinia			
carotovora C-3	83	33	50
atroseptica E-25	81	32	49
solanisapea	84	30	54
Escherichia			
coli BM12	87	39	48
coli B	82	38	44
paracolon	71	38	33
coli NRRL 210	73	37	36
coli Squibb #19	80	21	59
coli Squibb #1	46	31	15
Vibrio			
cholerae INABA	95	18	77
cholerae OGAWA	96	14	82
cholerae	78	13	75
Proteus			
anindologenes NRRL #B-418	78	23	55
mirabilis	67	23	44
mirabilis NRRL #B-400	68	15	53
morganii (U.Ch.)	67	11	56
ammoniae NRRL #B-420	58	11	47
vulgaris NRRL #B-123	59	8	51
vulgaris X19 (U.Ch.)	69	3	66

Control contains cells, distilled water, acid to pH 3.5. Controls showed no lysis.

* Lysozyme dilution 1:60,000.

† Calculations: *S. bareilly*.

	Optical density (2-log G)	
	Lysozyme suspension	Lysozyme-free suspension (control)
A. Original suspension	0.523	0.523
B. After addition of lysozyme acid and 1 hour incubation	0.215	0.208
C. After addition of alkali to B	0.040	0.168
D. Total lysis (clearing) for this species expressed as percent of potential clearing	$\frac{(0.215-0.040)}{0.215}$ =81%	
E. Proportional changes:		
(1) Due to lysozyme	$\frac{(0.215-0.040)-(0.208-0.168)}{0.215-0.040}\times 0.81$ =62%	
(2) Due to alkali	81 −62 =19%	

trast. That autolytic changes were minimal in *Salmonella bareilly* and of considerable proportions in *Proteus morganii* is apparent. In the control tubes a drop in the optical density was consistently observed with most species. That this should occur in the absence of lysozyme is a phenomenon yet to be explained. *S. bareilly*, in comparison to *Pr. morganii*, showed a substantial clearing accountable to lysozyme. This type of observation was the basis of the screening procedure. Table 2 summarizes the results of certain strains tested from each genus. They are arranged in order of decreasing lysozyme sensitivity.

Differences in sensitivity were observed not only between genera but also between strains within a species group.

The most and least sensitive species comprise the limits of the range of lysis within the genus. This range and the average lysis of all the strains tested in each genus are graphically presented in figure 1. From these data three groups of sensitivity are apparent. These were arbitrarily considered as "sensitive" group I, "relatively sensitive" group II, and "insensitive" group III. The latter group is so designated because the percent of lysis was considered to be within experimental error. The order of decreasing lysozyme sensitivity was: group I—*Salmonella, Brucella;* group II—*Klebsiella, Shigella, Neisseria, Pseudomonas, Pasteurella, Erwinia, Escherichia;* group III—*Vibro, Proteus*. Generally, the lysozyme lysis followed the order of total lysis in each genus; however, three exceptions were noted. The pseudomonas species showed a higher total clearing than any other genus, but its lysozyme sensitivity was inter-

mediate in the spectrum. Vibrio and proteus strains were least sensitive to lysozyme. However, *Vibro*, demonstrated a total clearing second only to *Pseudomonas*, while *Proteus* exhibited the least amount of total lysis of all the genera screened.

The variations in the range of lysis due to lysozyme between and within the generic groups are notable. The salmonellas, for example, showed a sizable lysis range (32 to 78% of the observed lysis), while the escherichia strains demonstrated much less (18 to 38%). There were differences within each genus and likewise strain differences within each species. *Salmonella typhosa* #24 gave 72% lysozyme lysis, strain #55 gave 48%, whereas strain #25 gave 38%.

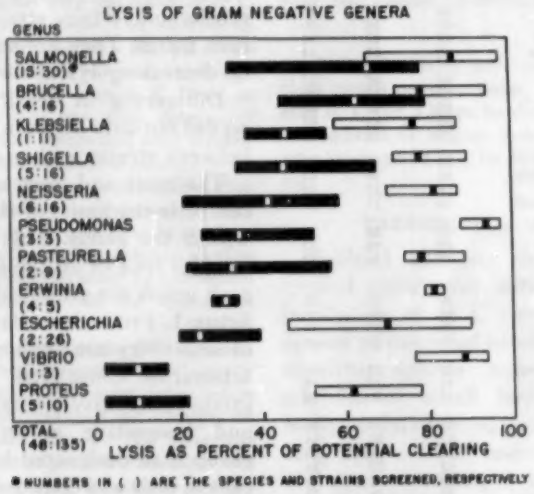

FIG. 1.—Lysis observed as total lysis (□) and lysozyme lysis (■) as proportion of total lysis. Averages: □ ■

Viability studies

Organisms located in the intermediate and near-the-top positions of the lysozyme spectrum were tested for their viability; the insensitive species were similarly exposed, then plated for comparison. This was done to correlate lysis with viability since some strains might be inhibited by the enzyme but not lysed appreciably. The results of a typical experiment are shown in figure 2.

It is evident that the lytic-resistant *Proteus vulgaris* strain was not killed to the extent of the lysable *Salmonella montevideo* strain. *Salmonella cholerasius* var. *Kunzendorf*, *Shigella ceylonensis*, *Shigella alkalescens*, and *Proteus mirabilis* were intermediate, respectively, in the killing spectrum. The viability studies, therefore, show a similar spectrum to that of the lytic studies. Irrespective of the dilution of lysozyme (1:3000 or 1:60,000), all species showed that a progressively higher percentage of the cells was killed when exposed for 3 hours than for 1 hour.

DISCUSSION

Impressions of the discrepancies recorded in the earlier literature relative

to the insensitivity of the gram-negative bacteria to lysozyme may be explained on the basis of impure lysozyme sources, or inadequate methods for measuring bacteriolysis, or the inability to obtain optimum conditions for the lysis of the microorganisms. To obviate these difficulties in our experiments, spectrophotometric measurements of lysis of cells exposed to solutions of pure lysozyme, followed by the application of the Nakamura technique, were found to be adequate for demonstrating that the enzyme had had an effect on most species.

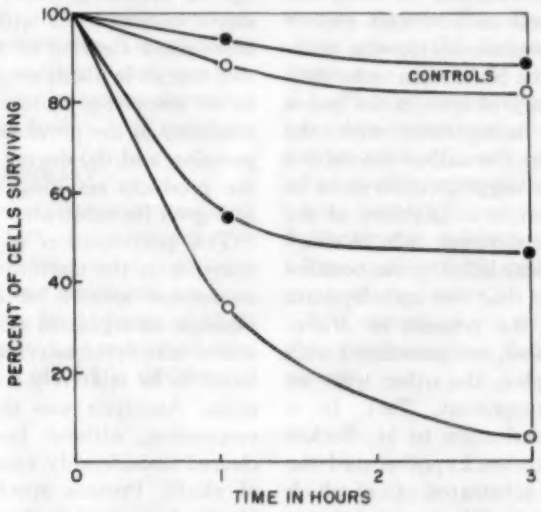

FIG. 2.—Surviving cells for *S. Montevideo* (— ○ —) and *Pr. vulgaris* (● — ●) at a lysozyme dilution of 1:3000. Controls were lysozyme free.

Nakamura[9] showed that acid and lysozyme were necessary to bring about lysis of certain gram-positive cells when alkali was added to the suspension. Congram[13] verified the necessity of these conditions and followed the gram reaction throughout the sequence of the lytic process. Gram-positive cells, sensitive to lysozyme, always became gram-negative whether or not they were solubilized. Evidence presented here showed clearly that the addition of alkali to pH 9.8 was necessary with every strain to demonstrate the action of lysozyme.

As with gram-positive species, the clearing of a cell suspension with this enzyme appears to involve adsorption, diffusion, substrate degradation and solubility. Autolytic enzymes, especially in *Vibro cholerae*, apparently contribute to the clearing phenomenon also. The demonstration that lysis did occur only when lysozyme was present, and to a greater extent at the higher enzyme concentrations with all the organisms tested, suggests that these bacteria do contain the substrate of lysozyme. The mucopolysaccharide substrate demonstrated by Epstein and Chain[12] in the gram-positive, lysozyme-sensitive bacteria was found by them only in traces

13. Congram, L. 1952, Master's Thesis, Purdue University.

in the gram-negative "resistant" species. Had these authors used the Nakamura technique, the evidence of clearing might have been more extensive in their so-called "resistant" strains and thus evidence of the substrate more clearly demonstrated. The same observation could apply to the very extensive studies of Venco[14] with pathogenic bacteria in the presence of tears or egg white. He concluded that all pathogenic gram-negative bacteria were insensitive to the enzyme. Vaccaro, Cabezas, and Copaja,[15] as well as Teti and Fiore,[16] drew similar conclusions, likewise without regard for the Nakamura technique.

The wide range of lysis in the genus *Salmonella*, in comparison with the limited range in the rather insensitive proteus strains, suggests differences in the quantity and/or availability of the substrate in the different cells. Webb,[17] working with heat-killed gram-positive cells, postulated that two carbohydrate substrates may be present in *Micrococcus lysodeikticus*, one associated with the gram complex, the other with an endocellular component, Teti, in a personal communication to M. Becker of these laboratories, hypothecated the presence of two substrates, one of which was somatic. Hartsell[18] suggested that a negative test for lysozyme lysis may be due to a lack of substrate; however, he noted that, although the substrate might be present, solubility conditions might not be optimal for clarification of the suspension following lysozyme action. Therefore, in this study we used standard conditions which were known to provide optimal solubility factors for many gram-negative bacteria.[9]

It should be noted, however, that differences in protein structure of the individual cells might also account for solubility variations in response to lysozyme action. It is not known whether globular or linear proteins compose the cells of gram-negative species. Whatever their form, the cross linkages could be broken by various agents, including lysozyme, if its substrate is complexed with protein. The subsequent clearing of the gram-negative species in alkali would depend then on the amount of protein disruption, the solubility of the previously formed acid proteins, and the degree of solubility of the products resulting from lysozyme acting on its substrate.

The participation of the autolytic enzymes in the clarification of the cell suspension should be considered. *V. cholerae*, an organism well known for its active autolytic enzyme system, was found to be relatively resistant to lysozyme. Autolysis was shown in a cell suspension, without lysozyme, which cleared considerably upon the addition of akalli. Proteus species showed an identical response to the enzyme, as did *Vibrio;* however, the former cultures did not clear as much in the lysozyme-free control when alkali was added. The presence of a minute amount of substrate in either of these organisms, as discused previously, would explain this correlation in lysozyme sensitivity. Autolytic enzymes active over the pH range 3 to 8 would account for the clearing of *Vibrio* and *Proteus* in the control tubes. Meyer and Hahnel[19] claimed that variations in the sensitivity of lysozyme of certain organisms were due to changes

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in the enzymes contained in the bacterial cell. The associative action of these enzymes with lysozyme over the pH range used in the Nakamura technique is yet to be evaluated.

The experimental observations reported here support the findings of Myerholtz and Hartsell[8] that the gram-negative group of bacteria, under certain experimental conditions, is sensitive to the action of lysozyme. The extent of lysis is proportional to the concentration of the enzyme, and the degree of sensitivity differs within genera, species, and even strains. The viability of salmonella, shigella, and proteus species exposed to lysozyme showed a spectrum similar to the lytic response.

SUMMARY

1. The evidence demonstrates that lysozyme will act on gram-negative cells if certain experimental conditions are provided. These conditions are that the cells be grown for 18 hours at 25 C, that they be exposed, as unwashed cells, to lysozyme at a pH 3.5 in dilutions of hydrochloric acid and incubated at 45 C for 1 hour, that $N/20$ sodium hydroxide be added to the suspension to pH 9.8 before the temperature becomes lower than 40 C. The effect of the enzyme would not be observed without these conditions.

2. The spectrum of lysis, in order of decreasing sensitivity to lysozyme and according to the extent of lysis, was: group I (sensitive)—*Salmonella*, *Brucella;* group II (relatively sensitive)—*Klebsiella*, *Shigella*, *Neisseria*, *Pseudomonas*, *Pasteuralla*, *Erwinia*, *Escherichia;* group III (insensitive)—*Vibro*, *Proteus*.

3. Viability studies showed that the lysozyme-insensitive species and certain species relatively easily lysed were not killed to the extent of the sensitive cultures; however, lysozyme had had a demonstrable inhibitory action. The killing and the lytic action were of the same relative order.

4. Concepts of the nature of lysis of the gram-negative bacteria are discussed.

TOXIC END PRODUCTS FROM *PASTEURELLA PESTIS*

I. A COMPARISON OF LYSATE TOXIN WITH THAT OBTAINED FROM THE ACTION OF BILE SALTS

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Toxins have been obtained from *Pasteurella pestis* by methods which fall into three categories. *Lysis*: Markl (1898 and 1900) used filtrates of old broth cultures. Girard (1939) employed a system of freezing and thawing. Jawetz and Meyer (1943) held agar-grown suspensions at 37 C for 48 hours and then at 4 C for 24 hours. *Desiccation:* Besredka (1905) used extracts from organisms dried by simple desiccation in vacuo. Rowland (1912) prepared toxins by extraction of organisms dried by mixture with anhydrous sodium sulfate. Quan, Chen, and Meyer (1950) used acetone for the desiccation. *Chemical extractions:* Lustig and Galeotti (1897) extracted organisms with weak potassium hydroxide. Somewhat similar alkaline extractions were employed by Geltenkov (1938).

These reports established the fact that end products can be derived from both living and killed bacterial cells which are toxic for some laboratory rodents, generally mice. It is by no means clear that all of these workers were dealing with the same end products or, in some cases, as to whether the toxin yields were great enough to suggest more than laboratory artifacts.

The present report deals with the release from *Past. pestis* of two types of toxins clearly distinguishable by various properties.

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METHODS AND MATERIALS

Culture.—Past. pestis, E.V. 76, "avirulent," obtained from Robic in Madagascar in 1951, was used throughout this work. This is the strain employed for human immunization (living cells) in the French colonies. For routine mass cultivation Blood Agar Base (Difco) was used in Roux bottles. Unless otherwise specified these cultures were maintained at 37 C for 72 hours, the organisms then being collected in distilled water.

Estimates of viable populations.—Serial dilutions of bacterial suspensions were prepared in brain heart infusion broth. Small samples (0.1 to 0.5 ml) of these dilutions were spread on the surfaces of blood agar base plates containing 0.0001% gentian violet. Duplicate plates were used. Colony counts were made on the 3rd day, and from these results the number of viable cells in the original suspension was calculated.

Toxin titrations.—All products were tested for toxicity for 17 to 20 g white mice, suitable dilutions in geometric series (4-fold) being injected intraperitoneally in 0.5 ml amounts. Each group consisted of 8 mice. Mice were observed for 10 days. The LD_{50} was calculated by the method of Reed and Muench. For purposes of comparison twice the reciprocal of the calculated LD_{50} obtained from 0.5 ml inoculums is considered as the number of "units" of toxin per ml.

Toxin neutralisation tests.—Serial 2-fold dilutions of toxin preparations were mixed with equal volumes of a serum diluted 1 to 25. After incubation at 4 C for 18 hours, the toxin-serum mixtures were injected intraperitoneally into mice in 1.0 ml amounts, using 8 mice per group. Observation was for 10 days. The LD_{50} of toxin was calculated for each titration and expressed as a dilution of the toxin. In each series there was included a titration with normal rabbit serum. From these was calculated the following expression:

$$\text{Neutralization index} = \frac{\text{reciprocal of toxin dilution end point with normal rabbit serum}}{\text{reciprocal of toxin dilution end point with unknown rabbit serum}}$$

Thus normal rabbit serum represents unity, and figures above this indicate greater neutralization.

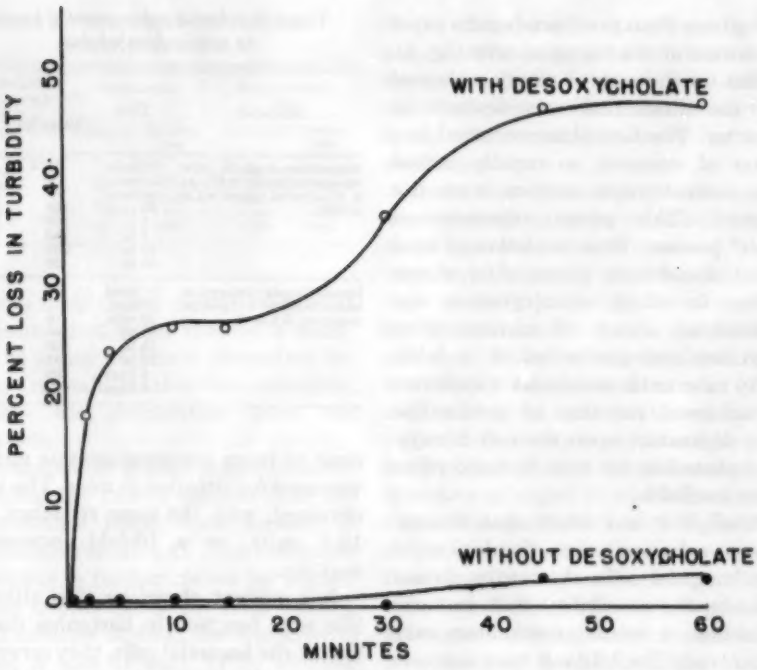

FIG. 1.—Enhancement of lysis of *Past. pestis* by sodium desoxycholate (0.5%).

RESULTS

Release of toxic end products following treatment with bile salts.—Inasmuch as bile salts hasten the rate and degree of lysis of certain bacteria it was reasoned that these substances might facilitate the production of a potent toxin with *Past. pestis*. Aliquots of bacterial suspensions were therefore mixed with equal amounts of various bile salts in 1% solutions. After 15 hours at 4 C the mixtures were centrifuged at 3000 r.p.m. for 60 minutes. Dilutions of the supernates were then injected. Controls showed that the bile salts alone were not toxic in the dilutions employed. The results of one such experiment are shown in table 1.

All bile salts employed gave rise to increased toxin yields. Sodium desoxycholate and sodium ricinoleate proved far superior to others. Under the conditions of this experiment, it is concluded that the bile salts either gave a greater release of toxin or produced an enhancement of toxicity for substances released, or both.

Effect of concentration of bile salts.—In another series of experiments graded amounts of sodium desoxycholate were added to constant amounts of bacterial suspensions. After 15 hours at 4 C the supernates gave the following results in toxin titrations (in each instance the values are for 1×10^9 cells): 0%—1.0 unit; 0.1%—22 units; 0.5%—33 units; 2.5%—70 units. Thus it is clear that a quantitative relationship exists between the amount of chemical added and the amount of toxic substance derived.

Enhancement of lysis of Past. pestis *by sodium desoxycholate.*—Microscopic and turbidimetric studies showed that the addition of bile salts to a suspension of

agar grown *Past. pestis* produced a rapid dissolution of the bacterial cells (fig. 1). Studies on the rate of reaction showed that the dissolution was diphasic in character. The first phase occurred in a matter of minutes, so rapidly indeed that accurate rate studies were not achieved. This phase represents a "flash" process. This was followed by a second dissolution phase of a slower nature, in which disintegration was initiated at about 30 minutes after admixture and proceeded at a fairly steady rate until maximum dissolution was achieved, the time of termination being dependent upon the cell density. No explanation for this diphasic effect is now available.

Although it is evident that desoxycholate enhances the dissolution of these bacterial cells, the results do not eliminate the possibility that in toxin production, a second mechanism may be involved. The bile salt may activate some precursor into a toxic form or it may convert a toxin into a substance of greater toxicity. In order to study this possibility desoxycholate was added to a cell free autolysate.

Enhancement of toxicity of autolysates by the action of bile salts.—An autolysate of *Past. pestis* was prepared by the method of Jawetz and Meyer (1943) and the superate filtered trhough a Berkefeld candle. The filtrate in this instance gave 1.6 toxin units/1 $\times 10^9$ cells. To the filtrate was then added an equal volume of 1% sodium desoxycholate. After 1 hour at room temperature this mixture was used for titration in mice. The value obtained, with the same reference, was 16.1 units, or a 10-fold increase in toxicity.

TABLE 1.—*The effect of added bile salts upon the recovery of toxin from suspensions of* Past. pestis.

Viable population before dilution = 2.4×10^{10}/ml		
Bile salt	Toxin units/ml diluted material	Toxin units/1 $\times 10^9$ viable cells
None	15.2	1.3
Sodium glycocholate	125	10.4
Oxgall (from dehydrated bile)	380	31.7
Sodium taurocholate	380	31.7
Sodium desoxycholate	1126	95.0
Sodium ricinoleate	1524	127.0

TABLE 2.—*Rate of enhancement of toxicity by sodium desoxycholate.*

Material	Time	Toxicity	
		Units/ml	Units/1 $\times 10^9$ cells
Suspension of viable cells; desoxycholate to 0.5% at 4 C (initial count = 2.6 $\times 10^{10}$)	Original (No desoxycholate)	13.5	0.5
	90 min	100	7.7
	3 hr	170	13.1
	5 hr	260	20.0
	20 hr	600	46.2
	46 hr	20	1.5
Lysate filtrate; desoxycholate to 0.5% at 4 C (initial count = 1.0×10^{10})	Original filtrate	64	6.4
	10 min	216	21.6
	24 hr	580	58.0
	48 hr	400	40.0
	72 hr	262	26.0
	5 days	200	20.0
	8 days	160	16.0

It is evident, therefore, that although bile salts function in hastening disruption of the bacterial cells, they serve also in the activation or conversion of some relatively nontoxic precursor into highly toxic substances either by alteration or by combination.

The time element in the effect of sodium desoxycholate.—In order to study the rate of alteration in toxicity by sodium desoxycholate two experiments were carried out. In the first experiment a suspension of organisms was treated with desoxycholate to a final concentration of 0.5%. The results of titrations for toxins in the supernates at various intervals thereafter are shown in table 2. It is evident that the alteration under these conditions involving whole cells requires a definite time in order to reach maximum levels, that is, from 20 to 30 hours at 4 C, but that thereafter the degree of toxicity declines rapidly.

In a second experiment desoxycholate was added to a lysate of *Past. pestis* to a final concentration of 0.5%. Titrations at various intervals gave results as shown in table 2. In this instance the in-

TABLE 3.—*Comparative heat stabilities of toxin preparations.*

Temperature of Heating (30 min.)	Autolysate type		Desoxycholate type	
	Units/ml	Percent loss	Units/ml	Percent loss
None	242		562	
44 C	212	12	137	76
51 C	160	34	<10	>96
58 C	80	67	<10	>96
65 C	<3	>99	<10	>96

crease in toxicity was clearly evident at 10 minutes but again reached a maximum at about 24 hours, thereafter declining gradually, rather than abruptly, as with the preparation from the whole cells.

Thermolability of toxin preparations.—The foregoing results suggest that the desoxycholate toxin, once formed, is not stable, even at 4 C. This difference in stability is further shown by studies on heat lability (table 3). With heating for 30 minutes at 44 C the lysate toxin showed a loss of only 12%, whereas the loss with desoxycholate toxin was 76%. At 51 C the desoxycholate toxin level was below the point of estimation, whereas the autolysate type toxin showed a loss of only one-third of its original potency. These results again indicate a difference in the nature of the toxic end products with the two types of preparations.

Formation of desoxycholate toxin from heated suspension.—In the foregoing experiment the great sensitivity of the desoxycholate-type toxin was demonstrated. If it is assumed that this toxin is derived from a precursor it is of interest to determine the effect of heat upon the source material with reference to its toxin-yielding potentiality. The results of such an experiment are shown in table 4. It will be noted that bacterial cells heated to 67 C still gave significant toxin yields when treated with sodium desoxycholate, although as previously shown, the two types of toxin are completely inactivated at this temperature. If the concept of a precursor is employed, the results would indicate that the precursor is far more heat stabile than the toxin itself. It also shows that living cells are not necessary for toxin production.

Comparison of toxin preparations as to time of death in mice.—All animals in these experiments were observed for 10 days, but it was soon apparent that each type of toxin preparation was marked by characteristic death distribution patterns. Thus, mice inoculated with the desoxycholate toxin were found dead within 24 hours, whereas deaths from the lysate toxin continued to occur for a period of days. Experiments were therefore arranged in which the time of death was carefully recorded. In each of the two experiments which are combined in table 5, parallel groups of mice were inoculated with nearly equivalent amounts of the respective toxins; in the first series 3.5 and 3.75 units or LD_{50}'s per mouse, in the second series 7.5 and 7.9 units.

By close observation it was noted that mice which succumbed at an early hour first became drowsy at about 60 minutes after inoculation. Just before death they became hyperirritable and, if stimulated, underwent violent convulsive seizures. If unstimulated, death occurred without outward change, and the animal remained in a seemingly normal stance. This applied to all mice receiving desoxycholate toxin in these amounts.

Late deaths were preceded by an entirely different picture. By 6 hours after inoculation the mice showed

TABLE 4.—*Toxin values after treatment of heated suspensions with sodium desoxycholate.*

Temperature of heating (30 min.)	Viable population/ml	Toxin yields, units/ml	Percent of original potential
Unheated	2.6×10^{10}	1610	100
47 C	5.4×10^{9}	1014	63
57 C	80	468	29
67 C	0	364	23
77 C	0	<26	<1.6

ruffled fur and thereafter ceased to take food or water. Weight loss was pronounced.

The figures on time of death show that the two types of toxin can be distinguished clearly under these circumtances. In each instance all mice injected with desoxycholate toxin were dead within 13 hours, whereas with lystate toxin, mice continued to die for a number of days.

Immunologic studies on toxins.—In order to determine if the desoxycholate toxin might represent a substance with immunologic properties differing from that of the lysate toxin a series of rabbits was immunized with various antigenic preparations. The serums of these rabbits were then employed in neutralization tests.

Living *Past. pestis*, E.V., establishes a mild but usually nonfatal infection in rabbits. Repeated injections, as used in immunization, frequently continue to establish new infectious foci. The convalescent serums from such animals were shown to neutralize both types of toxins well although the degree was significantly higher for the desoxycholate toxin (cf. table 6). An immunizing suspension heated at 60 C for 30 minutes (heat-killed) gave high antitoxin against the lysate toxin but virtually none against desoxycholate toxin. Organisms heated to 80 C failed to engender antitoxins. This is of interest in connection with the matter of heat stability.

TABLE 5.—*Time distribution of deaths following inoculation of mice with lysate and desoxycholate-type toxin.*

	Type of toxin preparation			
	Lysate	Desoxycholate	Lysate	Desoxycholate
Toxin units injected:	3.75	3.5	7.5	7.9
Number of mice:	155	95	44	38
Hours				
3.2	1	1	0	32
5.0	8	57	39	63
7.9	9	35	25	5
12.6	13	7	18	
20	5		7	
32	6		2	
50	16		2	
79	14		2	
126	19		5	
199	8			

Figures shown are percentages of total deaths occurring in time period ending at designated hour after inoculation.

TABLE 6.—*Neutralization of toxins by serums of variously immunized rabbits.*

Immunizing preparation	Lysate toxin	Desoxycholate toxin
Normal rabbit serum	1.0	1.0
Living *Past. pestis*, E.V.	5.3	15.8
Past. pestis, E.V., 60 C 30′	8.9	*
Past. pestis, E.V., 80 C 30′	1.0	0.9
Lysate toxin, formalinized	3.5	1.6
Desoxycholate toxin, formalinized	3.3	9.2

Figures shown represent neutralization indices.

$$NI = \frac{\text{reciprocal toxin dilution end point with normal rabbit serum}}{\text{reciprocal toxin dilution end point with unknown serum}}.$$

* Irregular results in repeated titrations at various combination levels. End points not apparent and cannot be calculated.

The toxins themselves proved difficult as antigenic stimuli for their toxicity was too great in the desired amounts. Recourse was had to formalinized preparations. Immunization against lysate toxin gave fair antitoxin titers against the homologous toxin but little against the desoxycholate toxin. Formalinized desoxycholate toxin gave rise to excellent antitoxin levels against the same toxin and to good antitoxin against the lysate toxin.

From these results it appears that all desoxycholate toxins contain some lysate toxin but that preparations of the latter may contain little of the highly labile toxin.

These results may indicate that the highly labile toxin is in itself a rather distinct entity.

DISCUSSION

These experiments show that suspensions of *Past. pestis* treated with bile salts liberate substances of high toxicity for mice. Furthermore, it was shown that the addition of bile salts to cell-free lysates of this organism brings about great enhancement of toxicity

of the lysates. Two effects can be postulated. Firstly, as has been shown, the bile salts function in bringing about the dissolution of the cells. Secondly, the bile salts may either bring about the conversion of some poorly toxic substance into one of high toxicity or the activation of some nontoxic precursor into a highly toxic substance. It is not possible to clarify this question at this time.

Irrespective of mechanism of origin, the toxin produced by the action of bile salts (desoxycholate toxin) appears to be a substance of considerable lability. Since it is artificially produced it might be thought of as a laboratory curiosity. However, rabbits infected with "avirulent" strains of *Past. pestis* develop high antitoxin levels directed against this labile toxin. McCrumb et al (1954) demonstrated antitoxin against the labile toxin in the serums of persons convalescent from pneumonic plague. Other work, not yet reported, shows that monkeys recovered from infections with virulent organisms develop similar antitoxins.

The matter of activation of precursors into highly labile toxic substances is a subject of considerable interest. Our experience is that a number of conditions may produce some degree of activation but that none of these approaches the bile salts in degree of effectivity. These conditions are diverse in character and do not yet seem to fit into patterns.

Contrasting properties as between the more stabile lysate toxin and the highly labile desoxycholate toxin suggests that, although some relationship may exist, there is a considerable basis for speculation that they may be different compounds. Considering the fact that lysate preparations usually contain a small amount of labile toxin and that most desoxycholate preparations undoubtedly contain some lysate toxin, it is of interest that the experiments reported show, in addition to the factor of lability, a great divergence in pharmacodynamic effect and most suggestive evidence as to immunologic differentiation.

SUMMARY

The addition of bile salts to suspensions of *Pasteurella pestis* results in end products of high toxicity for mice. Similar end products were obtained by the addition of bile salts to cell-free lysates. These "bile salt" toxins are highly labile, and give differential symptomatic effects in mice. Immunologic studies suggest that the labile toxin may be distinct from the lysate toxin.

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STUDIES ON THE ANTIBODY PATTERNS IN PNEUMONIC PLAGUE PATIENTS

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The application of commonly employed serologic methods to the study of antibody production during the course of pneumonic plague has been delayed by the absence of effective therapeutic measures in this highly fatal disease. Furthermore, the difficulty of preparing standard reagents has complicated the elucidation of immunologic reactions in patients recovered from milder forms of bubonic plague. The simultaneous appearance of antibiotics effective in the control of pneumonic plague[1–3] and new and well standardized serologic procedures[4–7] have offered means for the elucidation of immunologic problems.

The present report is concerned with the study of antibody production during the course of pneumonic infection with *Pasteurella pestis* and an evaluation of the techniques used to demonstrate these antibodies.

MATERIALS AND METHODS

Serums employed in the investigation were obtained from patients with pneumonic plague treated in Madagascar between November, 1951 and April, 1952 under the auspices of the Pasteur Institute of Tananarive. The therapy of these patients has been reported in detail elsewhere.[3] The serum specimens from these patients were maintained in the frozen state except during transportation from Madagascar to Washington.

Bacterial-agglutination test.—A suspension of a virulent strain of *Past. pestis* (195/P), grown at 37 C, was inactivated with formalin, and repeatedly washed with saline to remove the excess soluble envelope substance.[4] Antigen-serum mixtures were incubated at 37 C for 2 hours, then transferred to 4 C for 18 hours. The highest dilution of serum causing visible agglutination was accepted as the titer.

Complement-fixation test.—This procedure has been described in detail by Chen et al[4] and may be outlined briefly as follows: Four units of antigen (fraction I, 1:500 dilution of 2 mg/ml) contained in 0.2 ml were added to equal quantities of serial 2-fold dilutions of previously inactivated serum and incubated for 4 hours at 4 C and 1 hour at 37 C in the presence of 2 units of complement. The addition of sensitized cells was followed by additional incubation at 37 C for 30 minutes.

Hemagglutination test.—The method of Landy and Trapani[8] using tannic acid-treated sheep

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TABLE 1.—*Titration of plague antitoxin in human serum.*

Serum			Dilutions of toxin				Calculated toxin LD_{50}	Calculated neutralization index
Source	Day	Dilution	1:10	1:40	1:160	1:640		
Case no. 7	2	1:10			1/8	6/8	1:352	100
Case no. 7	13	1:10		1/8	2/8	8/8	1:224	157
Case no. 7	36	1:10		0/8	5/8	8/8	1:120	290
Rabbit antiplague†		1:50	0/8*	7/8	8/8	8/8	1:22	

* Number of mice surviving/number of mice injected.
† Hyperimmune antiplague rabbit globulin (Lederle) employed as positive control.

erythrocytes subsequently sensitized with purified capsular antigen (fraction I) was employed. The human serums had previously been absorbed with sheep red cells. The highest dilution of serum producing complete agglutination of the coated erythrocytes was taken as the titration end point.

Toxin neutralisation.—Toxin used in the tests was the desoxycholate-type prepared by the method of Goodner et al.[9] In the present work, agar growths (72 hours, 37 C) were harvested and a sufficient amount of 10% sodium desoxycholate was added to give a final concentration of 1%. After 18 hours at 4 C, the preparation was centrifuged at 2600 r.p.m. for 30 minutes. The supernatant fluid was then decanted, distributed in 5 ml quantities, and stored at −20 C. Freshly thawed samples were employed for each neutralization test.

For the neutralization tests, serial 4-fold dilutions of toxin were mixed with equal volumes of the appropriately diluted serum to be tested and the mixtures held at 37 C for 1 hour prior to the injection of 1.0 ml amounts into the peritoneal cavity of each of 8 mice. The mice were observed for 10 days. The total quantity of serum available from the pneumonic cases was small, and for this reason more extensive titrations were not feasible. As a positive control, hyperimmune antiplague rabbit globulin (Lederle) was included in each test. This preparation has been shown to exhibit high antitoxic potency.[10] From the results obtained, the LD_{50} value of toxin in the presence of each serum was calculated by the method of Reed and Muench and expressed as a dilution of the standard toxin. All serum specimens from each patient were tested simultaneously. A comparison was then made with the serum obtained during the early and late phases of the illness and a relationship derived as follows:

$$\text{toxin neutralization index} = 100 \times \frac{\text{reciprocal } LD_{50} \text{ toxin titer with acute-phase serum}}{\text{reciprocal } LD_{50} \text{ toxin titer with convalescent-phase serum}}$$

In this manner, toxin-neutralizing activity can be expressed as percentage activity of the acute-phase serum. Thus, if the toxin titer with acute-phase serum was 1:256 and that of a convalescent-phase serum 1:100, the toxin neutralization index would be 100×2.56, or 256. An example of this type of analysis is shown in table 1.

Mouse-protection tests.—The passive-protective activity of these serums was measured by the technique described by Meyer and Foster,[8] modified in some instances because of limited quantities of serum. Mice were challenged subcutaneously with approximately 100 LD_{50}'s of virulent *Past. pestis* following the intravenous injection of 0.5 ml of undiluted serum to be tested. The protective index is expressed as the quotient of the percentage of mice that die divided by the average day of death. Thus, values from 10 to 0 represent increasing degrees of protection. Normal serum indices are usually 15 or greater.

Influenzal hemagglutination-inhibition.—The standard hemagglutination-inhibition test for influenza[11] as performed at the Army Medical Service Graduate School was employed using serum previously treated with *Vibrio cholerae* filtrate.[12] Each serum was tested with repre-

fied capsular antigen of *Pasteurella pestis*. Am. J. Hyg. **59**: 150–156.

9. Goodner, K., Pannell, L., Bartell, P. and Rothstein, E. L. 1954, Toxic end products from *Pasteurella pestis*. I. A comparison of lysate toxin with that obtained from the action of bile salts. J. Infect. Dis. **96**: 82–87.
10. Jawetz, E. and Meyer, K. F. 1944, Studies on plague immunity in experimental animals. I. Protective and antitoxic antibodies in the serum of actively immunized animals. J. Immunol. **39**: 1–14.
11. Hilleman, M. R., Mason, R. P. and Buescher, E. L. 1950, Antigenic patterns of influenza strains A and B. Proc. Soc. Exper. Biol. & Med. **75**: 829–835.
12. Hilleman, M. R. and Werner, J. H. 1953, Influence of non-specific inhibitor on the diagnostic hemagglutination-inhibition test for influenza. J. Immunol. **71**: 110–117.

TABLE 2.—*Neutralization of plague toxin with human serum.*

Case no.	Acute phase serum			Early convalescent serum			Late convalescent serum		
	Day	Toxin LD_{50}	Neutralization index*	Day	Toxin LD_{50}	Neutralization index	Day	Toxin LD_{50}	Neutralization index
4	3	1:256	100	14	1:132	194	37	1:208	123
5	3	1:208	100	14	1:120	173	37	1:240	87
6	2	1:256	100	13	1:256	100	36	1:100	256
7	2	1:352	100	13	1:224	157	36	1:120	290
10	2	1:240	100		Lost in Transit		30	1:256	94
13	6	1:120	100	18	1:80	150	32	1:120	100
14	6	1:288	100	18	1:184	155	32	1:288	100
18	2	1:240	100	11	1:120	200			

* Neutralization Index $= 100 \times \frac{\text{reciprocal } LD_{50} \text{ toxin with acute-phase serum}}{\text{reciprocal } LD_{50} \text{ toxin with convalescent-phase serum}}$.

sentative strains of influenza A, A′ and B; i.e., PR8, FW-1-50, and Motulsky, respectively. The serum titer is the highest initial dilution of serum causing complete inhibition of hemagglutination.

Cold agglutinins and streptococcus MG agglutinins.—These tests were performed according to the technique employed at the Army Medical Service Graduate School.[13] Washed human O erythrocytes (0.3 ml, 1%) are added to 0.3 ml of 2-fold serum dilutions and incubated at 4 C overnight for the detection of cold agglutinins. A previously standardized suspension of streptococcus MG is added to similar serum dilutions and read after overnight incubation at 37 C.

RESULTS

The results of this study may be divided into four categories depending on the antigen-antibody system involved.

1. *Toxin-neutralizing antibody* (tables 1 and 2).—Serum specimens from 8 patients were tested by this method. In all but one instance (case no. 10), a rise in antitoxin occurred during convalescence. The rise in neutralization index was never greater than 3-fold. Of 14 samples collected during convalescence, 9 showed definite increases in titer. A representative protocol is shown in table 1. In this instance, the figures have this significance: On the 13th day following onset, the patient's serum was able to neutralize 1.57 times as much toxin as was the serum on the 3rd day. By the 36th day, serum neutralizing capacity was nearly three times greater than during the acute phase. While sufficient analyses are not available for comparison with other serologic methods, it is interesting to note that in at least one instance, antitoxin was demonstrable in the absence of agglutinating and complement-fixing antibody (case no. 7).

2. *In vitro tests for antibodies produced against envelope substance* (table 3).—Serum specimens from 15 patients were tested by agglutination and complement-fixation and 9 demonstrated rises in antibody. Demonstrable antibody usually appeared between the 7th and 14th day of illness and serum titers as measured by both tests were comparable. Antibodies capable of agglutinating erythrocytes sensitized with envelope material appeared after the 6th day of illness in most instances. The time of appearance of these antibodies corresponded closely with the time of appearance of agglutinins and complement-fixing antibodies; however, hemagglutination titers were usually 20 to 40 times higher (extremes of 5 to 320 times the sensitivity were encountered) and this degree of increased sensitivity remained fairly constant in serial specimens from a given patient. Three pa-

13. Smadel, J. E. 1952, Serologic reactions in viral and rickettsial infections. *In* Viral and Rickettsial Infections of Man, edited by T. M. Rivers, Philadelphia, J. B. Lippincott, pp. 72–99.

TABLE 3.—*In vitro measurement of anticapsular antibodies.*

Patients				Serum specimens studied											
				Acute phase serum†				Early convalescent serum				Late convalescent serum			
No.	Hour Rx	Therapy*	*Past. pestis*+	Day	Agg.	CF	HA	Day	Agg.	CF	HA	Day	Agg.	CF	HA
1	8	C-S	+	3	4	0	ND	7	8	2	40	38			1,280
2	16	C	+	5	0	0	0	17	Lost in Transit			32	0	0	160
3	7	C	−	2	0	0	0	13	32	8	160				
4	9	C	+	3	0	0	0	14	64	32	5,120	37	64	116	5,120
5		C	−	3	0	0	10	14	32	64	1,280	37	32	64	1,280
6	9	C	+	2	0	0	10	13	0	0	640	36	32	0	160
7	7	C	+	2	0	0	0	13	0	A.C.	320	36		A.C.	160
9	3	C	+	2	0	0	0	9	0	0	0	31	0	0	0
10	3	T	+	2	0	0	0	22			320	30	32	8	320
11	24	T-S	+	3	0	0	0	18	32		320				
13	24	S	−	6	8	4	QNS	18	128	256	5,120	32	64	128	2,560
14	12	S	+	6	0	0	10	18	256	512	10,240	32	256	512	10,240
15	16	S	+	6	0	0	0	18	64	64	2,560	32	32	64	2,560
17	4	S	−	Not obtained				13			40				
18	3	S	−	2			0	15			1,280				

* C=chloramphenicol, T=terramycin, S=streptomycin.
\+ *Past. pestis* isolated from sputum.
† Agg.=bacterial agglutination.
CF =complement-fixation.
HA =hemagglutination.

tients (no. 2, 7, 9) from whose sputum *Past. pestis* was isolated failed to demonstrate serological evidence of plague infection by bacterial agglutination or complement-fixation. Antitoxin was measured in only one of these patients (no. 7) and a rise in this antibody was demonstrated (table 2). Hemagglutination failed to provide serologic confirmation of plague infection in one instance only (case no. 9). This patient was treated with chloramphenicol 3 hours after the onset of fever and although plague bacilli were isolated from her sputum, pneumonitis was never demonstrable roentgenographically. By comparison, therefore, the hemagglutination test appeared to be of greater value in the diagnosis of plague infection in this group of patients.

Since serum was not collected after the 38th day of disease, nothing is

TABLE 4.—*Mouse protection tests with human serums.*

Case no.	Period of disease					
	Acute phase (2–3 days)		Early convalescence (9–18 days)		Late convalescence (30–36 days)	
	Day	Index	Day	Index	Day	Index
2					32	9.7
3			13	3.1		
4			14	0	37	0
5	3	16.6				
6	2	9.1 (1:2)*	13	0	36	0
7			13	7.1		
9			9	15.9	31	2.5
10					30	3.6
11	3	14.1 (1:2)*	18	0		
12	2	13.7				
13			18	0	33	0 (1:4)*
14					33	0 (1:10)*
15			18	5.7	32	0
17			13	6.3		
18	2	13.2	15	0		

Results shown are mouse protection indices. $\frac{\text{Percentage of mice dead}}{\text{average day of death}}$ = mouse protection index.

If all mice survived the result is indicated as 0. Normal human serums gave 18.1, 15.4.

* Dilution of serum employed because of shortage.

known of the persistence of any of these antibodies; however, as demonstrated in table 3, a parallel decline in antibody titer as measured by these three tests had occurred by the 5th week of disease in one instance (case no. 13). A decrease in hemagglutinating antibody alone was also evident by the 5th week in cases 6 and 7.

3. *Mouse-protective antibodies* (table 4).—Serum shortages precluded the extensive use of this procedure. Certain data were derived from the study of 23 serum samples. None of the 5 acute phase serums gave protection. In the early convalescent phase 6 to 12 samples gave significant degrees of protection; in the later convalescent stage 7 of 8 were highly protective. More significant are the few instances of paired serums in which definite rise is indicated.

4. *Viral antibodies.*—The association of *Past. pestis* with another bacterium or respiratory virus in the production of pneumonic plague has been suggested by several investigators.[14–16] Relative to this concept, certain serologic procedures were employed to test for the participation in these cases of influenza viruses or the agents responsible for primary atypical pneumonia. Evidence that any of these agents was involved in the illness in question was not obtained. Rises in influenza antibodies were not demonstrated, and in one case only (case no. 4) was a slight increase in titer of cold agglutinins and streptococcus MG agglutinins observed.

DISCUSSION

Recovery from pneumonic plague following the use of chemotherapy is associated with the appearance of circulating antibodies which may be demonstrated by a variety of methods. The previously highly fatal nature of the untreated form of this infection precluded the possibility of studying antibody production in the unmodified illness, and it is therefore impossible to evaluate the effect of antibiotics on the immune reaction. Several concepts are, however, worthy of consideration.

The production of any of these antibodies may be curtailed by the mere limitation of antigenic mass since the antibiotic quickly suppresses the multiplication of plague organisms. If this were a major factor, one would expect a correlation between the extent of the infection prior to therapy and the ultimate amount of antibody produced. This phenomenon has been studied in infections caused by other microorganisms[17,18] and the suppression of antibody by early antibiotic therapy has been clearly demonstrated. The short duration of illness, which rarely exceeds 48 hours and is frequently as short as 24 hours in the untreated patient with pneumonic plague, precludes the possibility of comparing antibody response in early and last treated patients in this

14. Nicolle, C. and Gobert, E. 1924, Considerations generales sur la peste pulmonaire et sa nature. Arch. Inst. Pasteur, Tunis. **13**: 212–235.
15. Girard, G. 1927, Epidemiologie de la peste pulmonaire a Madagascar. Bull. Soc. path. exot. **20**: 233–251, 632–652.
16. White, N. F. 1923, Prevalence of epidemic diseases and port health organization and procedure in the Far East. Geneva League Nat. C. H. 130.
17. Brink, W. R., Rammelkamp, C. H., Jr., Denny, F. W. and Wannamaker, L. W. 1951, Effect of penicillin and aureomycin on the natural course of streptococcal tonsillitis and pharyngitis. Am. J. Med. **10**: 300–308.
18. Denny, F. W., Wannamaker, L. W. and Hahn, E. O. 1953, Comparative effects of penicillin, aureomycin and terramycin on streptococcal tonsillitis and pharyngitis. Pediatrics, **11**: 7–14.

disease. However, it may be noted that one patient (no. 9), who was treated prior to the appearance of roentgen evidence of pneumonitis, failed to develop any antibody demonstrable by the methods employed.

Although anticapsular antibodies are produced rather readily in the experimentally infected host, limitation of antigenic stimulation has been shown to alter markedly the production of toxin-neutralizing antibody.[10] This apparent difference in antibody-producing capacity by envelope antigen on the one hand and the toxic component on the other might explain the small amounts of antitoxin demonstrable in these serums (table 2), since repeated stimulation by toxin shown necessary to produce antitoxin in animals was not attained in these patients. In addition, the "antitoxic" qualities of the broad spectrum antibiotics, as demonstrated by Quan, Chen and Meyer,[19] may be a contributing factor in this regard. These authors have provided evidence that aureomycin, terramycin and, to a lesser extent, chloramphenicol are able to neutralize the effect of small amounts of plague toxin.

Although there is some variation in the titer of antibodies as measured by agglutination, complement-fixation, and hemagglutination, the difference is thought to be primarily one of sensitivity. Of the three serological procedures based on the reaction between capsular antigen and its antibody (bacterial agglutination, complement-fixation and hemagglutination), bacterial agglutination is the one most easily performed. Standardization of the antigen is less precise and specificity is therefore sacrificed. Nevertheless, agglutinating antigens, prepared from virulent organisms grown at 37 C, probably measure simply the anticapsular antibody content of any serum. The other two procedures have the distinct advantage of specificity since the reaction is limited by the use of a relatively pure antigen. Approximately 69% of the group was diagnosed in retrospect by agglutination or complement-fixation. Fortunately, these methods were diagnostic in three instances where *Past. pestis* was not isolated from the sputum (no. 3, 5, 13, table 3). However, sputum from three other patients yielded plague bacilli when subsequent serological evidence of plague infection was lacking. Hemagglutination of erythrocytes senstized with the protein capsular antigen proved to be the most sensitive method of detecting antibodies to *Pest. pestis* and was diagnostic in all but one case. While it is apparent that high titers of antibody per se are of little value, the increased sensitivity of any procedure may yield measurable antibody which is not detactable by other methods. For this reason, the more sensitive hemagglutination test might be the procedure of choice for a serological diagnosis of plague infection. Erythrocyte-sensitizing plague antigens have been described by both Amies[6] and Chen.[7] The degree of sensitivity described by Landy[8] and illustrated in this study was not achieved by either of the former authors and is presumably the result of tannic acid treatment of the erythrocytes. Furthermore, the antigen described by Chen is apparently a polysaccharide and devoid of the protective qualities of the capsular antigen, fraction I.

The role of plague toxin in the pathogenesis of plague remains obscure, and the significance of the appearance of toxin-neutralizing substance in the

19. Quan, S. F., Chen, T. H. and Meyer, K. F. 1950, Protective action of antibiotics against the toxin of *Pasteurella pestis* in mice. Proc. Soc. Exper. Biol. & Med. **75**: 548–549.

serum of convalescing patients is therefore not fully understood. The results do suggest that the toxin of *Past. pestis* is antigenic.

Finally, the appraisal of immune response is difficult in an endemic plague area where previous contact with plague infection or vaccines is possible. While vaccination with the EV strain of *Past. pestis* had not been practiced on a large scale in Madagascar for five years at the time of this study and individual histories failed to reveal any evidence of previous vaccination or infection in this group, the anamnestic effect of the present infection in any of these patients cannot be completely excluded.

SUMMARY

The work here reported has demonstrated that in pneumonic plague certain antibodies are developed as a result of the infection and in spite of early, intensive antibotic therapy. The techniques employed demonstrated the presence of antibodies against both the envelope substance of *Pasteurella pestis* and against the toxin component of this organism. Negative results were obtained as to the possible participation in pneumonic plague of influenza viruses or of the agents responsible for the production of cold agglutinins or agglutinins against streptococcus MG.

SPECIES SPECIFICITY OF THE ANTI-EGG PRECIPITIN IN SCHISTOSOME SERUMS

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It has previously been reported that when eggs of *Schistosoma mansoni* are incubated at 37 C in serums from humans infected with this parasite, a precipitate is formed contiguous to the shell (circumoval precipitate) which seems to be the result of the interreaction of specific substances in the egg with an antibody present in the serum.[1] The antibody is not absorbed by treating the serum with cercarial or with adult worm materials, but is absorbed when the serum is treated with egg. The antibody is still effective even after serum dilutions as high as 1:64, suggesting that anti-egg antibodies are present in high titer and that immunization by egg might represent a most important immunological response in chronic schistosomiasis.

This report indicates that the circumoval precipitin is not only stage specific, but that it is also species specific. Precipitate is not formed or else is formed only to a slight degree when schistosome eggs of one species are incubated in the serum from humans and animals infected with other species.

MATERIALS AND METHODS

The human and animal serums utilized in this study were obtained from the following sources:

1. *Schistosoma japonicum* antiserums from U. S. Army officers infected while working in the rice fields of the Davao Penal Colony, Davao, province of Mindanao, P. I. The serums were obtained 2 to 6 years after exposure and were made available to us by Dr. J. F. Kent, Department of Serology, Army Medical Service Graduate School. Diagnosis had been made by visualization of typical ova in the stools.

2. *Schistosoma hematobium* antiserums were furnished by Dr. A. Michael Davies, Department of Hygiene, The Hebrew University Medical School, Jerusalem, from natives of Egypt, Iraq, Morocco and Israel with history of long standing infections. Eggs of *S. hematobium* had been found in biopsy material from urinary bladders; laboratory examinations revealed no other schistosome infection.

3. *S. mansoni* antiserums from adult natives of Puerto Rico, where no other species of human schistosome is known to exist. Diagnosis was confirmed by either positive stool or rectal biopsy. Infections were probably acquired during childhood.

4. *Paragonimus westermani* antiserums from natives of the Shizuoka perfecture, Japan, were furnished by Dr. F. B. Bang and L. Senterfit, Johns Hopkins University, School of Hygiene and Public Health. Sputums from these patients had all been positive for eggs; the duration of infection varied from 3 months to 2 years.

5. Serums from 6 cows naturally infected with *Fasciola hepatica* in Puerto Rico, as confirmed by positive stools. The animals had had the infection for a minimum of 3 years.

6. Serums from mice and monkeys experimentally infected with *S. japonicum* or *S. mansoni* and hamsters infected with *S. hematobium*.

7. Serums from rabbits artificially immunized with lyophilized eggs of *S. mansoni*. Each rabbit was injected with a total of 13.28 mg of lyophilized egg material (containing some liver substance) suspended in Freund's adjuvant given intramuscularly during a 3-week period. Serums were obtained 5 to 10 days after the last infection.

The eggs of *S. hematobium*, *S. japonicum* and *S. mansoni* were isolated from the livers and intestines of infected mice and hamsters furnished by Dr. W. H. Wright and Dr. E. B. Cram, from the Microbiological Institute, National Institutes of Health, Bethesda, Maryland. The intestines or livers were ground in a Waring blendor, the eggs recovered from the sediment after the suspensions were allowed to settle in 250 to 500 cc

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1. Oliver-González, J. 1954, Anti-egg precipitins in the serums from humans infected with *Schistosoma mansoni*. J. Infect. Dis. 95: 86–91.

TABLE 1.—*Circumoval precipitation about trematode eggs after incubation in serums from naturally infected humans.**

Antiserum	Antigen (egg species)					
	S. hematobium		*S. japonicum*		*S. mansoni*	
	Percent of eggs positive	Mean degree of reaction	Percent of eggs positive	Mean degree of reaction	Percent of eggs positive	Mean degree of reaction
S. hematobium	11.1	1+	0	0	8.1	1+
	34.0	1+	0	0	8.5	1+
	39.1	1+	0	0	1.0	1+
	26.8	1+	0	0	0	0
	38.9	1+	0	0	0	0
S. japonicum	0	0	35.2	4+	25.0	2+
	0	0	21.8	1+	3.6	1+
	0	0	20.4	4+	6.9	1+
S. mansoni	0	0	0	0	82.1	4+
	0	0	0	0	68.7	4+
	0	0	0	0	65.9	4+
	0	0	0	0	66.0	4+
	0	0	0	0	40.6	4+
	0	0	0	0	61.3	4+
Normal serum	0	0	0	0	0	0

* No precipitation was formed about eggs of *F. hepatica* after incubation in all serums, nor about schistosome eggs of all species incubated in the *F. hepatica* and *P. westermani* antiserums.

conical sedimentation flasks. The majority of eggs recovered from all species were living (with active miracidia).

Eggs of *F. hepatica* were recovered from adult flukes placed in physiological saline and were stored in a similar 0.85% salt solution at 6 C until needed. For rapid embryonation the eggs were transferred to distilled water at room temperature; the water was changed daily and well developed miracidia were generally present by the 8th day.

Microscopical preparations were made by placing together 0.01 cc of each serum (inactivated at 56 C for 30 minutes) and the trematode egg suspension on a slide, and covering with a 22×22 mm cover glass rimmed with vaseline. In some instances two or more species of eggs were incubated in one type of antiserum. The preparations were examined after 18 to 24 hours incubation at 37 C and the percent of eggs showing precipitate and the degree of precipitation (1+ to 4+) were recorded.

One hundred and sixty-nine preparations were made with the various species of schistosome eggs incubated in all antiserums; the number of eggs per preparation varied from 13 to 118. On the vast majority of preparations (78.7%), the number of eggs were within the range of 21 to 73. The *F. hepatica* eggs were so numerous that only 100 eggs were examined from each preparation.

TABLE 2.—*Circumoval precipitation about trematode eggs after incubation in serums from experimentally infected and artificially immunized animals.*

Antiserum			Antigen (egg species)					
			S. hematobium		*S. japonicum*		*S. mansoni*	
Agent	Source	Method	Percent of eggs positive	Mean degree of reaction	Percent of eggs positive	Mean degree of reaction	Percent of eggs positive	Mean degree of reaction
S. hematobium	Hamster	Infection	28.7	2+	0	0	15.8	1+
			40.1	3+	0	0	6.5	1+
			38.1	3+	0	0	6.3	1+
S. japonicum	Mice	Infection	0	0	36.6	1+	10.5	1+
					34.6	4+	5.8	1+
S. mansoni	Mice	Infection	0	0	0	0	70.4	4+
S. mansoni	Rhesus monkey	Infection	0	0	0	0	54.1	4+
			0	0	0	0	63.1	4+
S. mansoni	Rabbit	Artificial immunization with egg	0	0	0	0	50.0	4+
							41.5	3+
None	Mice	None	0	0	0	0	0	0

FIG. 1.—Egg of *S. haematobium* incubated in serum from human infected with this parasite (2+ reaction). ×350.

FIG. 2.—Egg of *S. japonicum* in serum from human infected with this parasite (4+ reaction). ×400.

RESULTS

Incubation of the eggs of *S. hematobium*, *S. japonicum* and *S. mansoni* in homologous antiserums resulted in massive precipitation around eggs (tables 1 and 2, fig. 1 through 6). However, when eggs were incubated in heterologous antiserums, circumoval precipitate

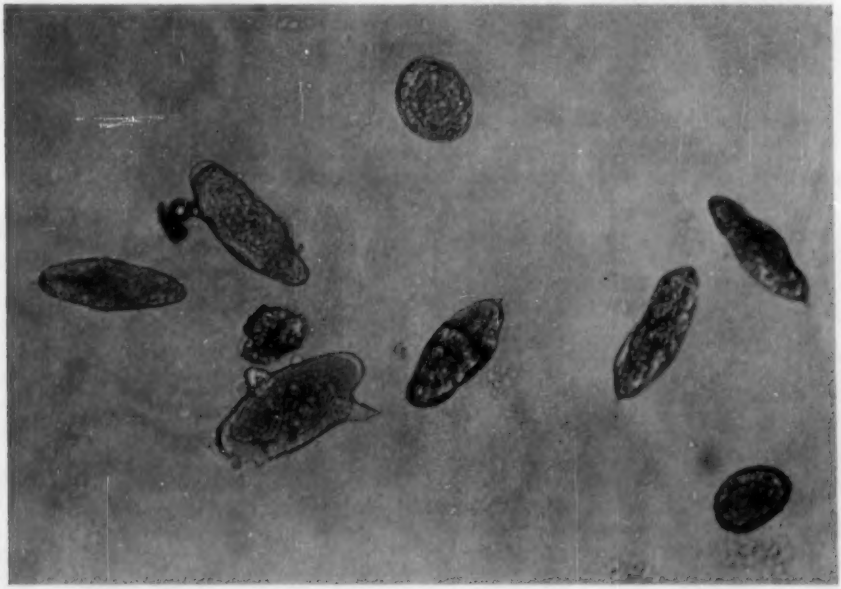

FIG. 3.—Precipitation about eggs of *S. mansoni* but no reaction about eggs of *S. japonicum* or *S. haematobium*, after incubation in *S. mansoni* antiserum. ×150.

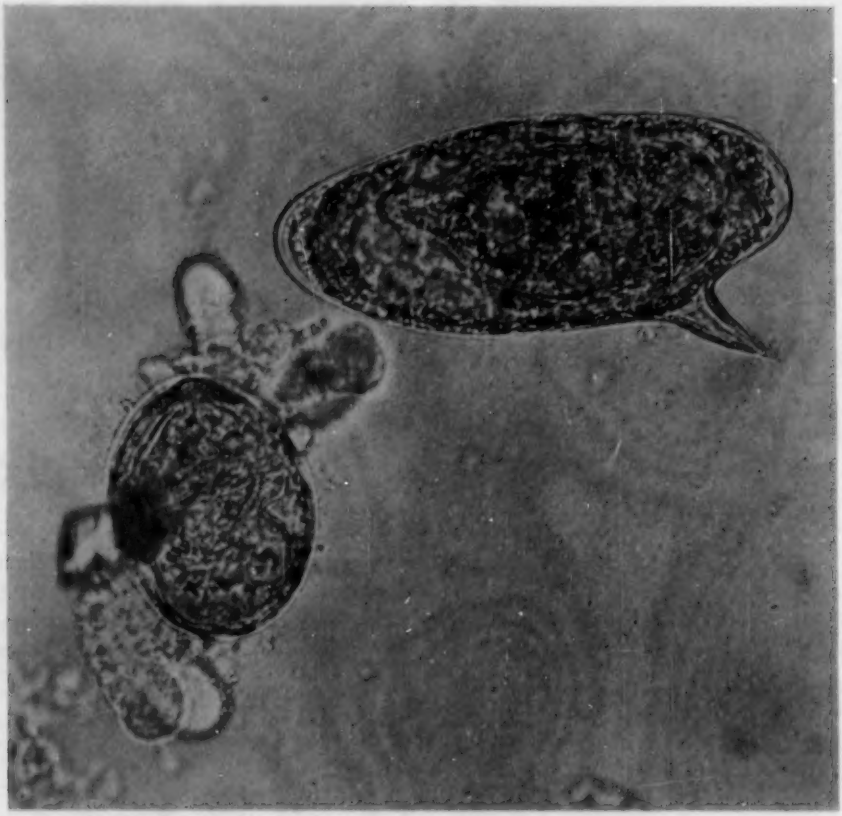

FIG. 4.—Precipitation about egg of *S. japonicum* but no reaction about egg of *S. mansoni* after incubation in serum from person infected with *S. japonicum*. ×500.

FIG. 6.—Egg of *S. mansoni* incubated in serum from persons infected with this parasite (4+ reaction). ×200.

FIG. 5.—Egg of *S. mansoni* incubated in serum from persons infected with this parasite (2+ reaction). ×300.

was only observed about eggs of *S. mansoni* incubated in *S. hematobium* and *S. japonicum* antiserums; these precipitates were much less massive and affected a smaller percentage of eggs than did the homologous antiserums (tables 1 and 2).

Results obtained with the antiserums prepared in experimental animals were similar. No circumoval precipitate was observed about *S. hematobium* and *S. japonicum* eggs incubated in *S. mansoni* antiserum from infected mice or monkeys or in serums from rabbits artificially immunized with lyophilized *S. mansoni* eggs, but precipitate was observed about *S. mansoni* eggs incubated in antiserums prepared in animals against the other two schistosome species.

The *F. hepatica* (from cattle) and *P. westermani* (human) antiserums all failed to react with trematode eggs of all species tested (tables 1 and 2).

The normal human and animal serums gave negative reactions on eggs of all species.

DISCUSSION

Many previous studies have shown the immunological interrelationship among parasites of the trematode group. Such distantly related forms as the lung fluke of frogs, *Pneumoneces medioplexus*, and free living trematodes such as *Planaria* can furnish antigen which elicit positive skin reactions in individuals infected with *S. mansoni*.[2] Extracts of the liver fluke *F. hepatica* can be used as antigens for complement fixation and skin test in schistosomiasis.[3]

Within the schistosomal group, the human species have so far been completely interchangeable as antigen sources for diagnostic tests. No chemical extraction nor physical dissection of adult trematodes has as yet resulted in the immunological demonstration of species specificity. The results obtained in this study indicate that there is definitely species specificity in the egg stage of the schistosomes, and permits for the first time a serological identification of the infecting organism. The fact that the reaction is absolutely specific when *S. hematobium* or *S. japonicum* eggs are used as antigen while there is a minor crossing with heterologous antiserums when *S. mansoni* eggs are used as the antigen, does not interfere with the use of this test diagnostically, since the precipitation is much more massive and affects a much larger percentage of the eggs when the antibodies were evoked by *S. mansoni* as the homologous agent.

The specific reaction of antiserums was further verified when two species of schistosome eggs were incubated in one type of antiserum. Negative reactions were observed on eggs of *S. hematobium* and *S. japonicum* when each of the species was incubated together with *S. mansoni* eggs in *S. mansoni* antiserum (fig. 3).

The reciprocity of the cross-reaction affords opportunity for phylogenetic speculation and, if the analogy to a viral agent is justified, reminds one of the immunological response against older strains of influenza A virus evoked by immunization with the new variants and the failure of any demonstrable antibodies to appear against the new variant when the animal is immunized by the older established strain.[4] If such anal-

2. Culbertson, J. T., Rose, H. W. and Oliver-González, J. 1947, Skin tests in *Schistosomiasis mansoni* with antigen from heterologous worms (Pneumoneces, Planaria). J. Infect. Dis. **80**: 218–221.
3. Culbertson, J. T. 1941, Immunity against Animal Parasites, Columbia University Press, pp. 210–211.
4. Hilleman, M. R., Mason, R. P. and Rogers, N. G. 1950, Laboratory studies on the 1950 outbreak of influenza. Pub. Health Rep. **65**: 771–777.

ogous reasoning is justified, this would imply that *S. mansoni* is the phylogenetic antecedent of both *S. hematobium* and *S. japonicum*.

The lack of cross reaction with *Fasciola* and *Paragonimus* is to be expected if species specificity pertains. The lack of circumoval precipitation with the homologous serum about *F. hepatica* eggs may correlate with the difference in the pattern of infection; in schistosomal infection, eggs are in physical position to serve as very effective antibody stimulators in contrast to the situation in liver fluke infections in which there is no tissue invasion by eggs and therefore inadequate antigenic stimulation for formation of antibodies.

The practical value of the circumoval precipitin reaction is indicated by the results obtained with one of the serums representing *S. hematobium* infections. This serum caused circumoval precipitation in 10.9% of *S. hematobium* eggs and 0% of *S. japonicum* eggs, but massive precipitation was formed on 48.3% of the *S. mansoni* eggs. These findings were taken to be indicative of *S. mansoni* infection, probably superimposed on *S. hematobium* infection. Further investigation of the clinical status by Dr. Davies revealed that this individual had been exposed together with others who were subsequently proven to have *S. mansoni* infections.[5]

SUMMARY

1. Living schistosome eggs become surrounded by precipitate when incubated at 37 C with homologous antiserums, both from natural and experimental infections, or artificial immunization.

2. This reaction is species specific with no cross reaction when *Schistosoma hematobium* or *Schistosoma japonicum* eggs are used as antigen, but there is a minor crossing when *Schistosoma mansoni* eggs are incubated in *S. hematobium* or *S. japonicum* antiserums.

3. No cross reaction could be demonstrated with the serums from humans infected with *Paragonimus westermani* or from cattle infected with *Fasciola hepatica*. No circumoval precipitate could be demonstrated when *F. hepatica* eggs were incubated with serums of infected cows, an observation which may correlate with the form of the disease in the mammal.

4. The merits of the circumoval precipitin test as a diagnostic procedure permitting specific diagnosis are discussed.

5. Frankl, O. 1953, Harefuah, 45: 85.

NOTE ON THE AGE AND SEX INCIDENCE OF POLIOMYELITIS

MILA E. RINDGE

A total of 366 cases of poliomyelitis was reported to the Connecticut State Department of Health during 1953. In an analysis of these cases it was noted that 53.6% of the cases occurred in males. However, if the cases were broken into age groups, of the 227 cases under 15 years of age, 142 or 62.6% were male, while of the 138 cases known to be 15 or more years old, 85 or 61.6% were female.

In an attempt to compare this finding with previous years, the experience of the past 5 years was analyzed, with results shown in table 1.

This showed that in the poliomyelitis cases under 15 years of age the proportion of males and females had remained about the same during the 5-year period, although the total percentage of cases in the older age group had increased, and each year a higher percentage of this group was made up of females. Since the percentage of total cases made up of the older males remained about the same (1949—15.2%, 1950—13.2%, 1951—15.5%, 1952—14.3%, 1953—14.5%), it was apparent that almost all of the increase in percentage of the older age group was due to the increasing numbers of females in this group. It is generally accepted that pregnancy increases the susceptibility of women to poliomyelitis. Therefore, pregnant women were removed from the group. Correction was also made by multiplying the number of nonpregnant women by a population factor* since there are more females than males in the older age groups. See table 2.

The resultant percentages are shown graphically in figure 1. This is a statistically significant trend, indicating that even without considering pregnant women, poliomyelitis is selectively involving greater numbers of the older female group in the population in Connecticut.

An effort was therefore made to determine whether any specific age group is especially implicated in the increase of older women. The percentage of total cases occurring in specific age groups for both sexes was determined. Again pregnant women were removed from the total as well as from the appropriate

TABLE 1.—*Poliomyelitis, 1949–53. Percentage by age group and percentage by sex within age group.*

Year	Total cases	Under 15			15 and over		
		Percent of total	Percent male	Percent female	Percent of total	Percent male	Percent female
1949	653	71.1	58.2	41.8	28.9	51.9	48.1
1950	478	67.3	64.2	35.8	32.7	46.8	53.2
1951	303	65.0	61.9	38.1	35.0	44.3	55.7
1952	392	67.3	61.4	38.6	32.7	43.7	56.3
1953	366	62.7	62.6	37.4	37.7	38.4	61.6

TABLE 2.—*Poliomyelitis, 1949–53. Percentage of females in 15+ age group corrected for pregnancy and population make-up.*

Year	Males 15 and over	Females 15 and over	Females, less pregnant	Females, corrected for population	Total males and corrected females	Percent female
1949	98	91	79	76	174	43.7
1950	73	83	71	68	141	48.2
1951	47	59	50	48	95	50.5
1952	56	72	66	63	119	52.9
1953	53	85	73	70	123	56.9

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* Population factor. According to the 1950 census there were in Connecticut 444,168 males between the ages of 15 and 45, and 464,329 females. This gives a M/F factor of 0.9566.

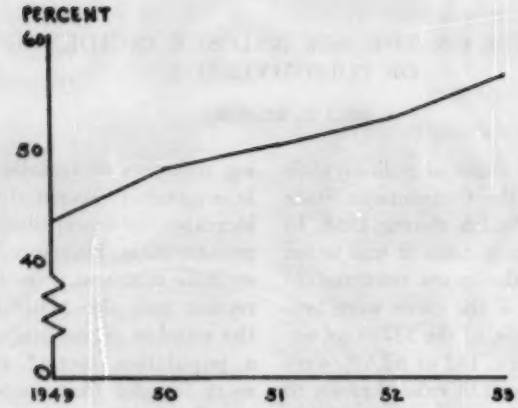

FIG. 1.—Poliomyelitis in Connecticut, 1949–1953. Percentage of females in 15 and over age group corrected for pregnancy and population make-up.

age group. Table 3 shows the resultant percentages.

It can be seen that the greatest increase is in the group of women 25 to 34 years of age. This almost parallels the increasing percentage of all women 15 or more years of age, as shown in figure 2.

It was suggested that the increase noted was largely among young mothers who had escaped infection in their own childhood, but whose children were bringing home unrecognized poliomyelitis and exposing their mothers to the disease. Adequate records on marital status are available only for 1953. In this year it was found that 62 of the females reported as having poliomyelitis were married. In the 1950 census there were 252,520 married women aged 15 to 40 in Connecticut. This would give a rate of 24.6 cases per 100,000 married women in 1953 in the state. The corresponding rate for married males (33 cases, 204,530 population) was 16.1.

Among the unmarried, 18 women 15 or more years old were reported, while the unmarried female population ages 15 to 40 numbered 140,040, giving a rate of 12.9 per 100,000. The corre-

TABLE 3.—*Poliomyelitis, 1949–53. Percentage by age group and sex, corrected for pregnancy.*

Sex	Age	1949		1950		1951		1952		1953	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Male	Under 5	86	13.4	58	12.4	33	11.2	41	10.6	37	10.4
	5–9	122	19.0	96	20.6	48	16.3	76	19.7	69	19.5
	10–14	62	9.7	52	11.2	41	13.9	45	11.6	36	10.2
	15–19	34	5.3	23	4.9	11	3.7	14	3.6	11	3.1
	20–24	17	2.6	13	2.8	12	4.1	9	2.3	5	1.4
	25–34	31	4.8	27	5.8	18	6.1	21	5.4	25	7.1
	35+	16	2.5	10	2.1	6	2.0	12	3.1	12	3.4
	Unknown	0		1	0.2	0		0		0	
Female	Under 5	68	10.6	33	7.1	22	7.5	34	8.8	22	6.2
	5–9	83	12.9	53	11.4	34	11.6	37	9.6	44	12.4
	10–14	43	6.7	29	6.2	19	6.5	31	8.0	19	5.4
	15–19	24	3.7	23	4.9	9	3.1	16	4.1	15	4.2
	20–24	19	3.0	15	3.2	11	3.7	16	4.1	12	3.4
	25–34	25	3.9	28	6.0	21	7.1	27	7.0	37	10.4
	35+	11	1.7	5	1.1	9	3.1	7	1.8	9	2.5
	Unknown	0		0		0		0		1	0.3
Total		641		466		294		386		354	

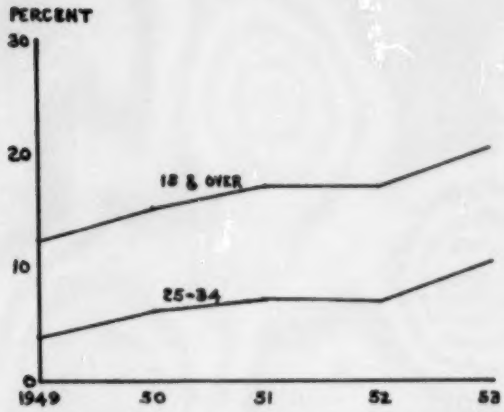

FIG. 2.—Poliomyelitis in Connecticut, 1949–1953. Percentage of females 15 and over and in 25 to 34 age group.

sponding rate for males (18 cases, 164,645 population) was 10.9

Thus it is evident that both married males and females in Connecticut had in 1953 a higher rate of poliomyelitis than did unmarried males and females, and that the rate in married females was significantly higher than in married males. Since 87.1% of the married women with poliomyelitis and 81.8% of the married men with poliomyelitis were recorded as having young children in the home, it would lend support to the idea that children are exposing their susceptible parents to the disease. The mother who spends more time with the child is more often exposed than the father.

It cannot be determined from data available whether this is a rising trend corresponding to the increase in female cases of poliomyelitis.

SUMMARY

It is well known that the percentage of poliomyelitis cases among people in the older age groups is higher than formerly. It is apparent that in Connecticut, at least, the increasing proportion of cases in the over 15 age group in recent years has been largely due to an increase in the number of females affected and that this increase is most marked among females 25 to 34 years of age. This is largely the young mother group who may have escaped infection in their own childhood and now may be infected by unrecognized cases of poliomyelitis among their children.

CORRECTION

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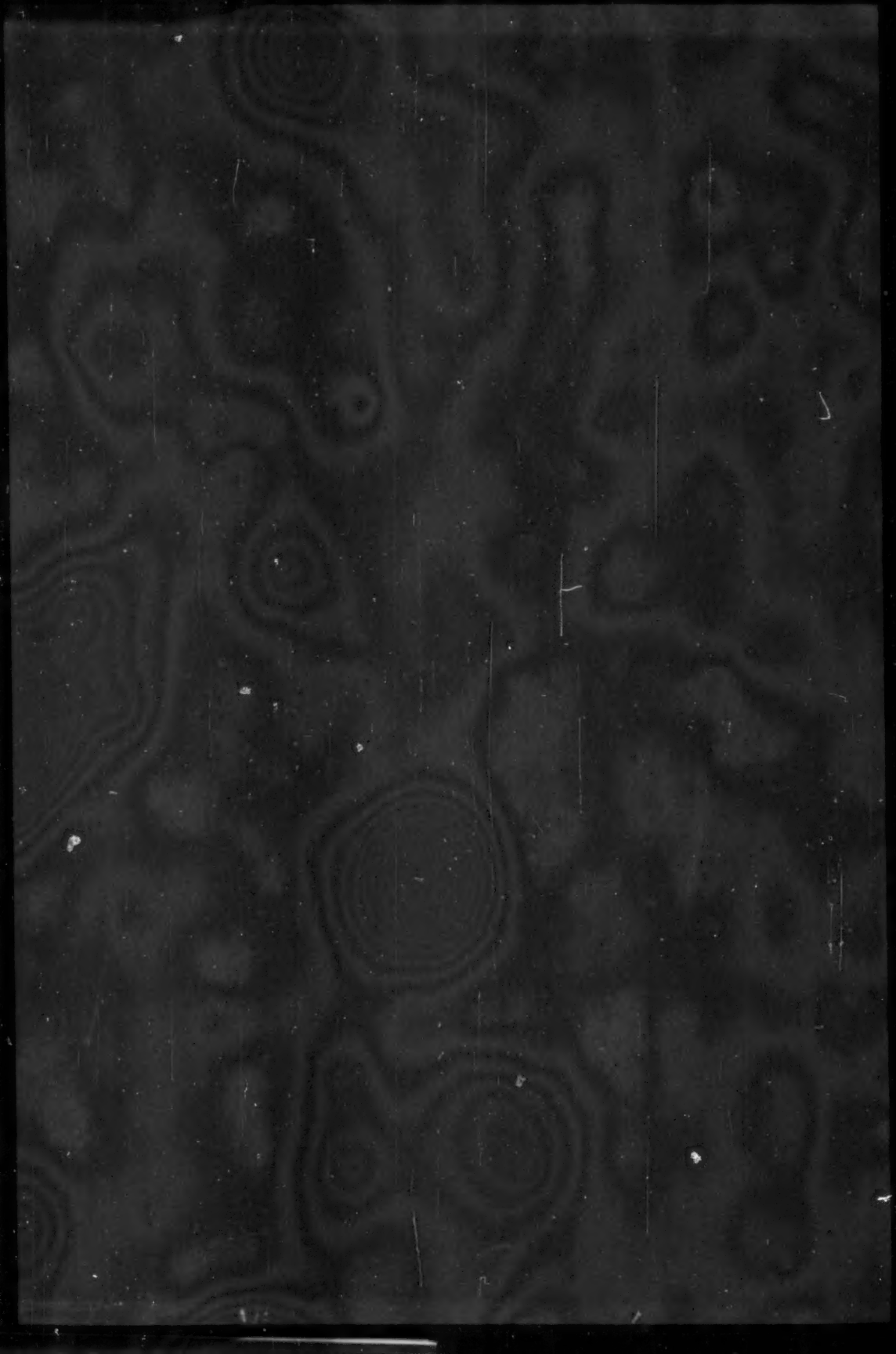
Page 215, 2nd column, line 25.—Change *Lept. icterohaemorrhagiae* to *Lept. grippotyphosa*.

Page 217, table 2.—In the 1st column, change albion to albino. Change the 6th, 7th, and 8th strains (3rd column) under albino mouse from Geffen to Meissar.



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